

# Improving Aspergillus carbonarius crude enzymes for lignocellulose hydrolysis

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# Improving *Aspergillus carbonarius* crude enzymes for lignocellulose hydrolysis

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**PhD Thesis**

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**Fungal Degradation**

# Preface

The present work represents the results of my Ph.D. study in the group Fungal Degradation, Department of Systems Biology, Technical University of Denmark in the period 15th of September 2012 to 14th of September 2015. Financial support for this project was provided through the Danish Council for Strategic Research in connection with the overall MycoFuelChem project (Grant no. 11-116803).

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## Summary

The primary aim of this thesis was to determine ways for enhancing the lignocellulose conversion potential of *Aspergillus carbonarius.* Approaches for enhancing the degradation of lignocellulose included: screening for fungal cellulase producers, media and growth optimization, genetic engineering and single enzyme supplementation. Fungal strains were screened in order to determine crude enzyme extracts that could be supplemented as boosters of *A. carbonarius* own crude enzyme extract, when applied in lignocellulose hydrolysis. The fungi originated from different environmental niches, which all had in common that they contained available lignocellulose as substrate. Fifteen different species were selected and in total 18 isolates were screened. Monoculture cultivations of these isolates were performed in both a solid and a liquid medium, for production of crude enzymes. The enzymes were analyzed for their potential in hydrolysis of wheat straw both by application of monocultures and by supplementing to crude enzymes of *A. carbonarius*. For the crude enzymes from solid cultivations there were eight isolates that showed synergistic interaction resulting in doubling and tripling of the glucose release in wheat straw hydrolysis. A completely different profile of synergy was observed for crude enzymes from liquid cultivations, as there were only three isolates that enhanced glucose release. Only one of these three isolates had shown synergistic effects when cultivated in a solid medium. The screening for synergistic activities should therefore utilize more than one medium for production of the enzymes to be investigated.

A more dedicated screening for cellulase producing fungi and potential boosters of *A. carbonarius* was also performed on selected indoor isolates. The indoor environment contains lignocellulose in the form of building materials. The fungal isolates screened were 21 common indoor fungi belonging to several genera. The most important finding of this screening was the overall strong enzyme profiles of both *Cladosporium sphaerospermum* and *Penicillium chrysogenum*. These two isolates had the highest endo-cellulase, β-glucosidase, mannase, β-galactanase and arabinanase activities, which makes them good candidates for crude enzymes to supplement *A. carbonarius* or for co-cultivation with it.

Based on a literature study of cellulase activities obtained for several ascomycetes in solid state fermentation (SSF) and submerged fermentation (SmF) it was found that SSF is a process that may yield higher enzyme titers. Therefore different SSF media and growth parameters were analyzed to determine the optimal strategy for *A. carbonarius* production of crude enzymes to be used in lignocellulose hydrolysis. Six different media were composed, all based on lignocellulosic waste as substrate. With regards to highest glucose release achieved in wheat straw hydrolysis by crude enzyme application, the most optimal medium was garden and park waste (GPW) supplemented by two nitrogen sources (GPW/N). The nitrogen sources

supplemented were urea and $(NH_4)_2SO_4$, and they were found to be essential for the enhanced enzyme production. An alternative nitrogen source in the form of municipal waste was added to the GPW medium, but it did not prove a good alternative. The GPW/N medium was found to yield optimal enzymes after cultivation for 5 days at 30 °C for both *Trichoderma reesei* Rut-C30 and for *A. carbonarius*. This was apparent although higher xylanase activity was observed after 5 days growth in a wheat bran/lactose medium and higher β-glucosidase activities were obtained after 14 days growth in a wheat bran sphagnum peat (WB/SP) medium. The results of this study emphasize that a sustainable method can be established for production of enzymes and potentially organic acids by using *A. carbonarius* grown on GPW.

A means to track the biomass production and thereby the growth of *A. carbonarius* in SSF was needed, as this could lead to better understanding of the potential growth rates on lignocellulosic substrates. Two indirect measurements of biomass were evaluated relying on the membrane associated enzyme, β-N-acetylhexosaminidase, and a membrane sterol, ergosterol. The biomass measurements were performed for a total of four fungi from the genera *Aspergillus*, *Trichoderma* and *Talaromyces* when cultivated in a solid WB/SP medium. There was unfortunately a large difference in the reproducibility of the measurements, with the enzyme having highest standard deviation of 11 - 23 % compared to the 2 - 8 % standard deviations of ergosterol measurements. Also it was primarily the ergosterol measurements that showed an increased biomass when comparing 7 and 14 days cultivations. According to the ergosterol measurements *Aspergillus saccharolyticus* and *A. carbonarius* produced the least amount of biomass relative to *T. reesei* Rut-C30 and *Talaromyces pinophilus*. From this study it was concluded that ergosterol rather than β-N-acetylhexosaminidase should be used for determining fungal biomass in SSF. It was also found that enzyme activity was not linearly correlated to the amount of Erg (biomass) measured.

In another study conducted during this Ph.D. project, the *A. carbonarius* crude enzyme extract was mixed with either lytic polysaccharide monooxygenase (LPMO) from *Podospora anserina* or *Thermoascus aurantiacus* in combination with a cellobiose dehydrogenase (CDH) from *Neurospora crassa*. The aim was to evaluate whether the oxidative degradation of cellulose in wheat straw could enhance the glucose release when at the same time applying crude enzymes of *A. carbonarius* in the hydrolysis. It was found that the addition of purified *P. anserina* LPMO and either CDH or ascorbate could not increase the glucose release in wheat straw hydrolysis. However, when a high concentration of the *P. anserina* LPMO, 4.21 mg/g cellulose (mg/g), was supplemented to the mixture of commercial enzyme preparations Novozym 188 and Celluclast 1.5L a slight but significant increase in glucose was observed. A large increase in glucose release was observed for the supplementation of *T. aurantiacus* LPMO, both at the high concentration of 4.21 mg/g and at the low concentration of 0.21 mg/g. The glucose release rose from 5.92 to 7.2 or 7.3 g/L. This

corresponds to an improvement of the cellulose conversion by 6.8 %. The activity of the LPMO was not enhanced by addition of CDH, but when adding ascorbate to the high concentration of LPMO a significant boosting effect was observed resulting in the highest glucose release of 7.63 g/L. Indeed this was the most optimal improvement of *A. carbonarius* crude enzyme hydrolysis potential that was achieved corresponding to a total cellulose conversion efficiency of 32 %. Thereby it was shown that a strong boosting of the crude enzymes could be obtained by a single enzyme, the *T. aurantiacus* LPMO, and that there is a large difference in the effect depending on the origin and type of the LPMO. The effect of LPMO depends on the amount of cellulases applied as well, as it was found that supplementing Ta_LPMO to Celluclast 1.5 L and Novozym 188 gave an increased efficiency of 25.9 %.

Finally it was analyzed whether the same *P. anserina* LPMO and the CDH from *N. crassa* would show an improvement of the crude enzymes when heterologously expressed in *A. carbonarius*. The resulting mutants constitutively expressed LPMO and CDH, however, there was no improvement of the glucose release obtained from wheat straw. Despite the lack of effect on glucose release, a significant effect was though observed for xylose release in hydrolysis of wheat straw. The improvement in xylose release ranged from 0.83 g/L to 1.2 g/L. Therefore the addition of LPMO from *P. anserina* and CDH from *N. crassa* may still boost the hydrolysis of wheat straw with regards to hemicellulose conversion, which may make the cellulose more accessible.

The results obtained during this Ph.D. project successfully represent strategies by which *A. carbonarius* hydrolysis of lignocellulose may be improved. The successfully applied strategies were blending of monocultures and optimization of cultivation conditions. By optimization of cultivation conditions it was further shown that this fungus can be employed to utilize lignocellulosic waste in the form of GPW for effective production of enzymes. The most potent improvement of *A. carbonarius* crude enzymes was achieved by the supplementation of a single LPMO enzyme from *T. aurantiacus*. For *A. carbonarius* growth on or conversion of lignocellulose a boosting can therefore be achieved by supplementing with LPMO.

# Sammenfatning

Hovedformålet med denne afhandling var at bestemme måder til at forbedre lignocellulose omdannelses potentialet af *Aspergillus carbonarius*. Tilgangene til at forbedre nedbrydningen af lignocellulose inkluderede; screening efter svampe der kan producere cellulaser, medie og vækst optimering, genetisk modificering og supplering med enkelte enzymer. Svampe stammer blev screenet for at afgøre hvilke rå enzym ekstrakter der kunne blive suppleret, som forstærker, af *A. carbonarius* egne rå enzym ekstrakt når det bliver anvendt i lignocellulose hydrolyse. Svampene stammede fra forskellige miljømæssige nicher, der alle havde det til fælles at de indeholdte tilgængeligt lignocellulose som substrat. Femten forskellige arter blev udvalgt og sammenlagt blev 18 isolater screenet. Monokultur dyrkninger af disse isolater blev udført i både et fast og et flydende medie, til produktion af rå enzymer. Enzymerne blev analyseret for deres potentiale i hydrolyse af hvede strå ved både at anvende monokulture og ved at supplere dem til rå enzymer fra *A. carbonarius*. For de rå enzymer fra faste dyrkninger var der otte isolater der viste synergistisk interaktion, resulterende i fordobling og tredobling af glukose frigørelsen i hvede strå hydrolyse. En helt anderledes profil af synergi blev observeret for rå enzymer fra flydende kulturer, da der kun var tre isolater der forøgede glukose frigørelsen. Kun et af de tre isolater havde vist synergi effekt ved dyrkning i et fast medie. Screeningen efter synergistiske aktiviteter bør derfor gøre brug af mere end et medie til produktion af enzymerne der skal screenes.

En mere målbevidst screening efter cellulase producerende svampe og potentielle forstærkere af *A. carbonarius* blev også lavet for udvalgte indendørs isolater. Indendørs miljøet indeholder lignocellulose i form af bygge materialer. Svampe isolaterne der blev screenet var 21 almindelige indendørs svampe tilhørende flere slægter. De vigtigste resultater fra denne screening var at både *Cladosporium sphaerospermum* og *Penicillium chrysogenum* havde en overordnet stærk enzym profil. Disse to isolater havde de højeste endo-cellulase, β-glucosidase, mannase, β-galactanase og arabinanase aktiviteter, hvilket gør dem til gode kandidater for rå-enzymer til at supplere *A. carbonarius* eller til co-kultivering med denne.

Baseret på litteratur studier af de opnåede cellulase aktiviteter for adskillige ascomyceter i fast medie fermenteringer (FMF) og flydende fermenteringer (FF) blev det fundet at FMF er en process der kan yde højere enzym koncentration. Derfor blev forskellige FMF medier og vækstbetingelser analyseret for at bestemme den optimale strategi for *A. carbonarius* produktion af rå-enzymer til anvendelse i lignocellulose hydrolyse. Seks forskellige medier blev sammensat, alle baseret på lignocellulose affald som substrat. Med hensyn til den højeste glukose frigivelse opnået ved anvendelsen af rå enzymer i hvede strå hydrolyse, så var det mest optimale medie have og park affald (HPA) suppleret med to nitrogen kilder (HPA/N). De

supplerede nitrogen kilder var urea og $(NH_4)_2SO_4$, og det blev observeret at de var essentielle for den øgede enzym produktion. En alternativ nitrogen kilde i form af husholdnings affald blev tilsat til HPA mediet, men det viste sig ikke at være et godt alternativ. HPA/N mediet blev observeret til at give det optimale enzym udbytte efter kultivering i 5 dage ved 30 °C for både *Trichoderma reesei* Rut-C30 og for *A. carbonarius*. Dette var tydeligt, selvom en højere xylanase aktivitet blev observeret for 5 dages vækst i et hvedeklid/lactose medie og højere β-glucosidase blev opnået efter 14 dages vækst i et hvedeklid sphagnum tørv (H/ST) medie. Resultaterne af dette studie understreger at en bæredygtig metode kan blive etableret til produktionen af enzymer og potentielt organiske syre ved brug af *A. carbonarius* groet på HPA.

Det blev fastlagt at en metode til at følge biomasse produktionen og dermed væksten af *A. carbonarius* i FMF var nødvendig, da dette kunne lede til en bedre forståelse af potentielle vækstrater på lignocellulose substrater. To indirekte målinger af biomasse blev evalueret, beroende på det membran associeret enzym, β-N-acetylhexosaminidase, og en membran sterol, ergosterol. Biomasse målingerne blev udført for totalt fire svampe, fra slægterne *Aspergillus*, *Trichoderma* og *Talaromyces* efter kultivering i det faste H/ST medie. Der var uheldigvis en stor forskel i reproducerbarheden af målingerne, med NAHA enzymet havende den højeste standard afvigelse på 11 – 23 % i forhold til 2 – 8 % standard afvigelse for ergosterol målingerne. Desuden var det primært ergosterol målingerne der viste en forøget biomasse når man sammenlignede 7 og 14 dages kultiveringerne. I følge ergosterol målingerne havde *Aspergillus sachharolyticus* og *A. carbonarius* produceret den laveste mængde biomasse relativt til *T. reesei* Rut-C30 og *Talaromyces pinophilus*. Fra dette studium kunne det konkluderes at ergosterol rettere end β-N-acetylhexosaminidase bør blive brugt til bestemmelse af svampe biomasse i FMF. Desuden blev det bestemt at enzym aktivitet ikke kan korreleres linært til mængden af Erg (biomasse) målt.

I et andet studie udført undervejs i Ph.D. projektet blev *A. carbonarius* rå enzym ekstrakt blandet med enten lytisk polysaccharide monooxygenase (LPMO) fra *Podospora anserina* eller *Thermoascus aurantiacus* i kombination med en cellobiose dehydrogenase (CDH) fra *Neurospora crassa*. Formålet var at evaluere hvorvidt den oxidative nedbrydning af cellulose i hvede strå kunne forbedre glukose frigørelsen, når der samtidigt anvendes rå enzymer fra *A. carbonarius* i hydrolysen. Det blev observeret at tilsætningen af oprenset *P. anserina* LPMO og enten CDH eller ascorbate ikke kunne forøge glukose frigørelsen i hvede strå hydrolysen. Imidlertig kunne en lille, men signifikant forøgelse, i glukose frigivelse observeres når en høj koncentration af *P. anserina* LPMO, 4,21 mg/g cellulose (mg/g), blev suppleret til blandingen af de kommercielle enzym præparater Novozym 188 og Celluclast 1.5 L. En stor forøgelse af glukose frigørelsen blev iagttaget for suppleringen af *T. aurantiacus* LPMO, både ved den høje koncentration af 4,21 mg/g og ved den lave koncentration af 0,21 mg/g. Glukose frigørelsen steg fra 5,92 g/L til 7,2 eller 7,3 g/L. Dette

svarer til en forbedring af cellulose omdannelsen på 6,8 %. Aktiviteten af denne LPMO blev ikke forbedret ved tilføjelse af CDH, men ved tilsætning af ascorbat til den høje koncentration af LPMO observeredes en signifikant forøgelse resulterende i den højeste glukose frigivelse på 7,63 g/L. Netop dette var den mest optimale forbedring af *A. carbonarius* rå enzym hydrolyse potentiale der blev opnået, tilsvarende en total cellulose omdannelses effektivitet på 32 %. Derved blev det vist at en stærk forøgelse af rå enzymer kunne opnås ved tilsætning af et enkelt enzym, *T. aurantiacus* LPMO'en, og at der er en stor forskel i effekten afhængigt af oprindelsen og typen af LPMO.

Endeligt blev det analyseret hvorvidt den samme *P. anserina* LPMO og CDH'en fra *N. crassa* ville vise en forbedring af de rå enzymer når de blev heterologt udtrykt i *A. carbonarius*. De resulterende mutanter udtrykte LPMO og CDH konstitutivt, men der var ikke nogen forbedring af glukose frigørelsen opnået fra hvede strå. Selvom der var en manglende effekt på glukose frigivelsen, var der dog en signifikant effekt observeret for xylose frigivelsen i hydrolyse af hvede strå. Forbedringen af xylose frigivelsen vekslede mellem 0,83 g/L til 1,2 g/L. Derfor kan tilsætningen af LPMO fra *P. anserina* og CDH fra *N. crassa* stadig forstærke hydrolysen af hvedestrå med henblik på hemicellulose omdannelse, som kan gøre cellulosen mere tilgængelig.

Resultaterne opnået igennem dette Ph.D. projekt repræsenterer succesfulde strategier til forbedring af *A. carbonarius* lignocellulose hydrolyse. Strategierne som blev anvendt successfuldt var blanding af monokulture og optimering af kultiverings betingelser. Ved optimering af kultiveringsbetingelserne blev det endvidere vist at denne svamp kan blive brugt til udnyttelse af lignocellulose affald i form af HPA til effektiv produktion af enzymer. Den mest virksomme forbedring af *A. carbonarius* rå enzymer blev opnået ved tilsætning af det enkelte LPMO enzym fra *T. aurantiacus*. Til vækst på eller omdannelse af lignocellulose kan *A. carbonarius* derfor forstærkes ved supplering med LPMO.

# List of publications

## Work not included in this thesis

- Zoglowek M, Hansen GH, Lübeck PS, Lübeck M. Fungal consortia for conversion of lignocellulose into bioproducts. Chapter 12 in Fungal Biotechnology for Biofuels. Bentham Science Publishers. (**Tentative publication date is October, 2015**)

## Work included in this thesis:

- Andersen B, Poulsen R, Hansen GH. Cellulolytic and xylanolytic activities of common indoor fungi. Int Biodeterior Biodegradation. (**Submitted**)

- Hansen GH, Lübeck M, Frisvad JC, Lübeck PS, Andersen B. Production of cellulolytic enzymes from ascomycetes; comparison of solid state and submerged fermentation. Process Biochem 2015; 50: 1327-1341. (**Published**)

- Hansen GH, Poulsen R, Svenssen DK, Nielsen KF, Andersen B. Fungal enzyme production and biomass determination in solid state fermentation. J Ind Microbiol Biotechnol. (**Submitted**)

- Hansen GH, Nødvig CS, Ishida T, Samejima M, Igarashi K, Frisvad JC, Lübeck M, Andersen B. LPMO increase glucose and xylose yield in wheat straw hydrolysis when supplemented to crude enzymes of *Aspergillus carbonarius*. Biores Technol. (**Submitted**)

# Abbreviations

| | |
|---|---|
| **A** | Avicel |
| **AA** | Auxiliary activity |
| **Ac** | *Aspergillus carbonarius* |
| **aSBP** | Alkaline extracted sugar beet pulp |
| **ATCC** | American type culture collection |
| **AUC** | Area under the curve |
| **Avi** | Avicel cellulases/avicelases |
| **$a_w$** | Water activity |
| **AZCL** | Azurine cross-linked |
| **B** | Bagasse |
| **BG** | β-glucosidase |
| **BP** | Banana peel |
| **BS** | Barley straw |
| **BX** | Birch wood |
| **C** | Carbon |
| **CAZy** | Carbohydrate active enzymes |
| **CB** | Corn bran |
| **CBH** | Cellobiohydrolase |
| **CBP** | Consolidated bioprocess |
| **CBS** | Centralbureau voor schimmelcultures |
| **CC** | Corn cob |
| **CCR** | Carbon catabolite repression |
| **CDH** | Cellobiose dehydrogenase |
| **CE** | Cellulose |
| **CEb** | Cellobiose |
| **CMC** | carboxy methyl cellulose |
| **CREA** | Creatine sucrose agar |
| **CRISPR** | Clustered regularly interspaced short palindromic **repeats** |
| **CS** | Corn stover |
| **CSp** | Corn stover powder |
| **CT** | Cassava tuber |
| **CYA** | Czapek yeast extract agar |
| **dB** | Delignified bagasse |
| **DG18** | Dichloran 18 % glycerol agar |
| **DNS** | 3,5-dinitrosalicylic acid |
| **DOE** | Department of energy |
| **dsWB** | Destarched wheat bran |
| **EC** | Enzyme class |
| **EG** | Endoglucanase |
| **egPP** | Esparto grass paper pulp |
| **EGR** | Elephant grass |
| **Erg** | Ergosterol |
| **FF** | Flydende fermentering |
| **FMF** | Fast medie fermentering |
| **FP** | Filter paper cellulase/FPase |
| **FPU** | Filter paper activity unit |
| **G** | Glucose |
| **gDNA** | Genomic DNA |
| **GH61** | Glycoside hydrolase family 61 |
| **GP** | Grape pomace |
| **GPW** | Garden and park waste |
| **HPA** | Have park affald |
| **HPAEC** | High performance anion exchange chromatography |
| **HPLC** | High performance liquid chromatography |
| **IUPAC** | International union of pure and applied chemistry |
| **KP** | Kinnow pulp |

| | |
|---|---|
| **LPMO** | Lytic polysaccharide monooxygenase |
| **LM** | Liquid medium |
| **MEA** | Malt extract agar |
| **MM** | Minimal medium |
| **MSB** | Mixed substrate biomass |
| **MX** | methylxanthine caffeine |
| **NAHA** | β-N-acetylhexosaminidase |
| **Nc_CDH** | CDH from *Neurospora crassa* |
| **NCIM** | National collection of industrial microorganisms |
| **ND** | Not determined |
| **nkat/ml** | Nanokatal pr. (/) milliliter enzyme extract or supernatant |
| **NRRL** | National center for agricultural utilization research |
| **NREL** | National renewable energy laboratory |
| **OH** | Oat husk |
| **OP** | Orange peel |
| **OPw** | Oil palm waste |
| **OSX** | Oat spelt xylan |
| **Pa_LPMO** | LPMO from *Podospora anserina* |
| **PASC** | Phosphoric acid swollen cellulose |
| **PDC** | Partially delignified cellulignin |
| **pNP** | Para-nitrophenyl |
| **pNPG** | Para-nitrophenyl-β-D-glucopyranoside |
| **QM** | Quartermaster culture collection |
| **RB** | Rice bran |
| **RH** | Rice husk |
| **RPB2** | RNA polymerase II second largest subunit |
| **RS** | Rice straw |
| **RSM** | Response surface methodology |
| **R.T.** | Room temperature |
| **S** | Sucrose |
| **SBP** | Sugar beet pulp |
| **SCB** | Sugarcane bagasse |
| **SCP** | Sugarcane pulp |
| **SD** | Standard deviation |
| **SEA** | Steam exploded aspen |
| **SEB** | Steam exploded sugarcane bagasse |
| **SEWS** | Steam exploded wheat straw |
| **SF** | Sorghum flour |
| **SHF** | Separate hydrolysis and fermentation |
| **SiSF** | Simultaneous saccharification and fermenation |
| **SmF** | Submerged fermentation |
| **SNA** | Spezieller nährstoffarmer agar |
| **SS** | Sugarcane straw |
| **SSCF** | Simultaneous saccharification and co-fermenation |
| **SSF** | Solid state fermentation |
| **T** | Temperature |
| **U/gs** | Enzyme activity unit pr. (/) gram substrate |
| **U/ml** | Enzyme activity unit pr. (/) milliliter enzyme extract or supernatant |
| **U/mg** | Enzyme activity unit pr. (/) miligram |
| **VW** | Vinegar waste |
| **V8** | V8 juice agar plates |
| **WB** | Wheat bran |
| **WB/SP** | Wheat bran and sphagnum peat |
| **WC** | Walseth cellulose |
| **WCE** | Whatman CC41 cellulose |
| **WS** | Wheat straw |
| **WSp** | Wheat straw powder |
| **Xyl** | Xylanases |
| **YES** | Yeast extract sucrose |

# Table of Contents

# Chapter 1
# Introduction

## 1.1 Importance of lignocellulose

Lignocellulose, as a raw material for the biotechnology industries is abundant and renewable, making it a sustainable choice as a feedstock for producing chemicals of interest and value. Lignocellulose can be obtained from agricultural, municpal and forestry wastes and thinnings, energy crops and waste paper. There are, however, vast differences in the composition of lignocellulose materials obtained from each of these examples. The variations are apparent for the three main constituents of lignocellulose which are lignin (5-30 %), hemicellulose (15-35 %) and cellulose (30-50 %) [1,2]. The purest sources of cellulose can be found in cotton, flax and chemical pulp consisting of 60-95 %, while soft and hardwoods contain approximately 45 % [2]. Wheat straw is a major lignocellulose waste source in Europe and one of the cheapest raw materials for a biorefinery process [3]. Its composition has been determined to be varying between 29-35 % cellulose, 26-32 % hemicellulose and 16-21 % lignin [4]. Other reports on the composition of lignocellulose in wheat straw have shown a content of 36.6 % cellulose, 24.8 % hemicellulose and 14.5 % lignin [5] compared to 35-40 %, 25-30 % and 10-15 % for each component respectively [6]. These differences may be due to seasonal variations, sample age or experimental procedures.

Of the three components, lignin is a recalcitrant barrier of mainly coniferyl, paracoumaryl and sinapyl alcohols. Cellulose is a linear polymer of glucose monomers, linked by β-1,4-glucosidic bonds, and every second glucose residue in the chain has an orientation that is flipped 180 ° along the longitudinal axis. Two repeating residues of glucose in the cellulose chain with shifting orientation is a cellobiose residue. Individual cellulose chains bond together via hydrogen bonds and van der Waals forces to form fibrils. These organized fibrils of cellulose are the predominant crystalline form that is found in nature, but a small amount is present in an amorphous (unorganized fibrils) form, which is more susceptible to enzymatic degradation. Hemicellulose is a polysaccharide consisting of both C5 and C6 sugars such as D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose glucuronic acids etc. Hemicellulose is also primarily linear, however, with short branches of different sugars [4]. These hemicellulose polymers crosslink to lignin, while being linked to cellulose polymers thereby forming microfibrils [7]. Both cellulose and hemicellulose can be hydrolyzed enzymatically to their monosaccharide components, which make up the starting point for bio-based commodities and fine chemicals. Eleven years ago, the American department of energy (DOE) made a forecast of the most valuable chemical building blocks that could be produced from sugars via biological or chemical conversion of lignocellulose [8]. The building blocks can subsequently be converted

into high-value chemicals or materials. The sugar based building blocks are 1,4-diacids (succinic, fumaric and malic acid), 2,5-furan dicarboxylic acid, 3-hydroxy propionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol, and xylitol/arabinitol [8].

Another major product that can be produced from hydrolyzed lignocellulose is biofuels, such as ethanol or hydrocarbon fuel derived from levulinic, formic or acetic acid [9]. In 2009 the Renewable Energy Directive stated that by 2020, 10 % of the total energy used for transportation should originate from renewable sources. This directive also stated sustainability criteria and to reach these targets, 2$^{nd}$ generation and 3$^{rd}$ generation biofuels will be necessary [10]. In October 2013 the world's largest bioethanol plant was opened in Crescentino, Italy, with the world leading enzyme producer, Novozymes A/S, as its main shareholder [11]. This biorefinery focuses mainly on the conversion of the local waste product wheat straw. There is though a lack of more large scale industrial biofuel plants in Europe, which could be due to challenges with financial viability, economies of scale and partly lack of political incentives.

For the production of either biofuel or chemicals, it is essential that the lignocellulose material is efficiently hydrolyzed to monomeric sugars. However, this is indeed a bottleneck due to the complexity and recalcitrance of lignocellulose, which makes it naturally resistant towards microbial attacks and enzymatic hydrolysis. Therefore, a pretreatment strategy needs to be developed in order to remove as much of the lignin and reduce crystallinity of cellulose while preserving the hemicellulose to make it more accessible to enzymatic hydrolysis. The dominant pretreatment approach starts with a mechanical processing (grinding/shredding) to increase surface area, followed by a physical and/or chemical step. Many parameters and operational conditions need to be balanced in order to achieve an optimal pretreatment. If conditions are too harsh, the sugars may be degraded and by-products can be formed such as furfural and hydroxymethyl furfural that inhibit the subsequent biological hydrolysis and fermentation [12]. If the conditions are not harsh enough, the accessibility of cellulose and removal of lignin may not be optimal. Following the pretreatment step is the enzymatic hydrolysis of polymeric cellulose and hemicellulose, to gain a maximum of monosaccharide sugars. Several enzymes are required, but among the most important for cellulose conversion are cellulases endo-glucanase (EG), exo-glucanase/cellobiohydrolase (CBH) and β-glucosidase (BG). Most of the important enzymes for lignocellulose hydrolysis are shown in Table 1.1. This step is of utmost importance for the yield of the following fermentation step, where the desired product(s) is(are) produced.

**Table 1.1** Main enzymes required for lignocellulose degradation.

| Substrate | Class of enzymes |
|---|---|
| Cellulose | Exo-β-1,4-glucan cellobiohydrolase (CBH) EC 3.2.1.91,<br>Endo-1,4-β-D-glucanase (EG) EC 3.2.1.4,<br>β-1,6-glucosidase (BG) EC 3.2.1.21,<br>Lytic Polysaccharide Monooxygenase (AA9) EC 1.-.-.-<br>Cellobiose dehydrogenase (CDH) EC 1.1.99.18, |
| Hemicellulose | Endo-1,4-β-xylanase EC 3.2.1.8,<br>Exo-1,4-β-xylosidase EC 3.2.1.37,<br>α-arabinofuranosidase EC 3.2.1.55,<br>Exo-β-mannanase EC 3.2.1.25,<br>Endo-1,6-β-galactosidase EC 3.2.1.23, |
| Lignin | Laccase EC 1.10.3.2,<br>Lignin peroxidase EC 1.11.1.14,<br>Manganese peroxidase EC 1.11.1.13. |

The enzymatic hydrolysis can be integrated in various ways in the overall process scheme in biorefineries. In general there are 4 main configurations that can be applied. In three of the configurations, the required cellulases and hemicellulases are produced in a separate step either on-site or acquired commercially [12]. This is done for separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SiSF) and simultaneous saccharification and cofermentation (SSCF) as shown in Fig. 1.1.

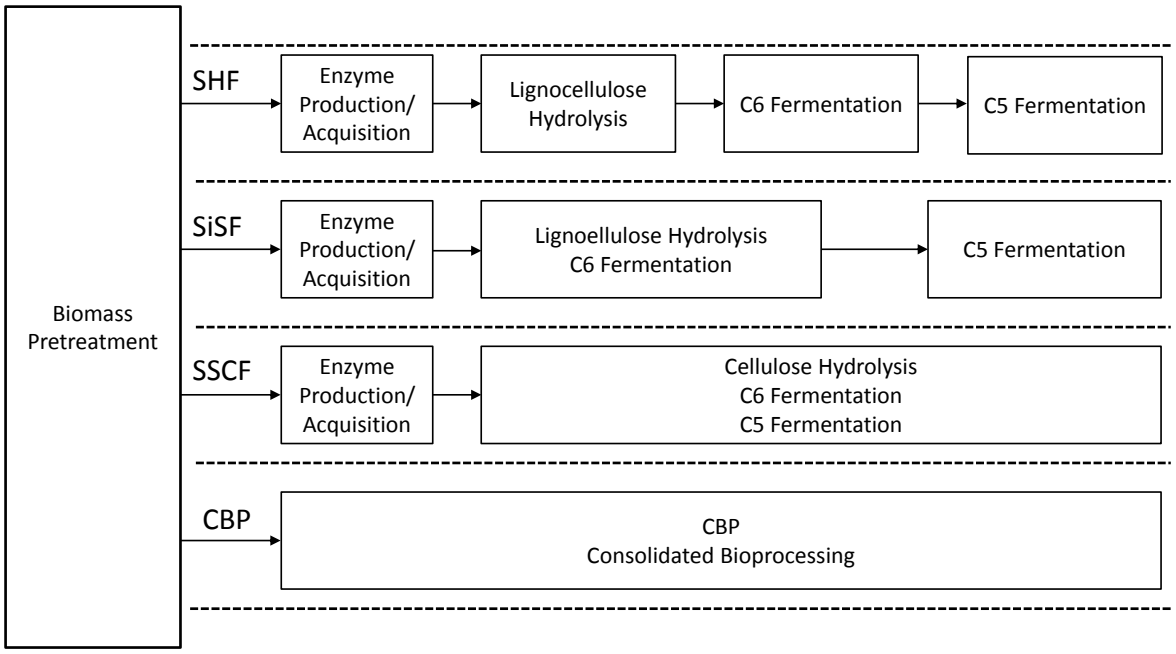


**Figure 1.1** Biomass processing schemes, where each box represents a bioreactor (not to scale). Processes represented are separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SiSF), simultaneous saccharification and cofermentation (SSCF) and consolidated bioprocessing (CBP). Adapted from [23].

The last configuration, consolidated bioprocessing (CBP), incorporates all process steps into one, by either using one organism for both production of enzymes, saccharification and fermentation of C6 and C5 sugars or utilizing co-cultivations for these steps [12]. The enzymatic hydrolysis is the most expensive step in the overall process owing to the cost of commercial enzymes, as these are projected to be 45 % of total processing cost [13]. In general enzymatic hydrolysis is considered as one of the most expensive unit operations along with pretreatment and feedstock acquisition, which can be offset by maximizing the conversion yield [14]. Commercially the main production organisms of cellulases and hemicellulases are ascomycetes of the genera *Trichoderma* spp. and *Aspergillus* spp. [15–18]. Although it is these two genera that are overrepresented in the commercial production of cellulases and hemicellulases, there are many other potential organisms of both bacterial and fungal origin that have been screened and analyzed for enzyme production [19–22]. The cost of the enzymatic hydrolysis could be lowered significantly by applying a configuration with on-site enzyme production, applying one of the many candidates that have already been screened.

On-site enzyme production can be achieved by 1) cultivation of multiple monocultures, followed by enzyme blending, 2) co-cultivation of compatible fungal strains in a single bioreactor, or 3) cultivation of a single strain genetically modified with several cellulolytic genes important for efficient biomass degradation. On-site production has been reported frequently using *T. reesei* as the cellulase producer, supplemented with β-glucosidase from *Aspergillus niger* or other Aspergilli [24–26]. Also on-site separate enzyme production was recently analyzed in lab-scale for *Trichoderma reesei* and *Aspergillus saccharolyticus* which yielded better results in hydrolysis of wet-exploded corn stover and loblolly pine compared to supplementing with *A. niger* [27]. In a recent study the promising β-glucosidase producer *Aspergillus carbonarius* was analyzed both in a mixed culture with *T. reesei* and as a monoculture blended with *T. reesei*. Monoculture blending and co-cultivation resulted in equally high efficiency of wheat straw hydrolysis [28]. In this thesis the main work was focused on optimizing the enzymatic hydrolysis potential of the promising β-glucosidase producing *A. carbonarius* with an application perspective as on-site enzyme producer or CBP organism in mind.

### 1.2 *Aspergillus carbonarius*

Recently the full genome sequence of *A. carbonarius* strain ITEM 5010 (Fig. 1.2), was generated and made available by the Joint Genome Institute (http://jgi.doe.gov/carbonarius).

**Figure 1.2** *A. carbonarius* cultivated (7 days) on wheat bran medium.

This strain was isolated from grapes in Italy in 2001, and is a known producer of ochratoxin A. However, the ochratoxin is only produced in a rich and easily metabolized culturing medium such as Czapek yeast broth, which mimics the high sucrose content of grapes [29]. Nevertheless *A. carbonarius* is much more than a toxin producer as shown by the high β-glucosidase production, as well as being a natural producer of citric acid similar to *A. niger* [30]. The potential of the genome has allowed for engineering of *A. carbonarius*, *e.g.* a deletion of the glucose oxidase led to increased organic acid production of both citric, oxalic and malic acid [31]. In general the available genome sequence supports efforts in optimizing expression and production of recombinant proteins as well as a sustainable chemical production [32]. Recently it has been reported that an efficient production of hydrocarbons with similarity to jet-fuel could be achieved by cultivation on a lignocellulose medium. It was found that out of 10 different carbon sources screened, oatmeal stimulated the best hydrocarbon (alkane/alkene) production [33].

With regards to enzyme production *A. carbonarius* has been shown to be an efficient β-D-xylosidase producer with a higher activity compared to *A. niger*, *A. nidulans* and *A. oryzae* when cultivated on wheat bran [34]. In a broad study of whether hydrolase profiles of black Aspergillus species could be used to characterize individual species, it was shown that *A. carbonarius* had a lower production of the screened enzymes when grown on wheat bran. There was a low activity of α-L-arabinofuranosidase, β-xylosidase, β-galactosidases and β-mannosidase, however, the β-glucosidase was approximately the same as that of 15 analyzed *A. niger* isolates [35]. The contradictions in the xylosidase activity that has been reported may be due to differences in the assay conditions or small differences in the cultivation conditions since they were incubated either 4 days (high acitivity) [34] or 2 days (low activity) [35]. In a more applied study, where *A. carbonarius* was also cultivated on wheat bran, the Avicelase and EG activities were 0.1 U/mL and 3 U/mL, respectively, which was approximately identical to *A. niger* [28]. In this study the authors also analyzed the

hydrolysis potential of *A. carbonarius* towards steam pretreated wheat straw, finding that the glucose release was approximately 7.5 g/L, while for *A. niger* it was 8.5 g/L. However, the potential of blending *A. carbonarius* monoculture or co-cultivating it with *T. reesei* Rut-C30 yielded 80 % hydrolysis efficiency, as it did when applying *A. niger* [28]. In this PhD thesis, the focus was on *A. carbonarius* due to the similar enzymatic profile of *A. carbonarius* and *A. niger*, the large potential of producing organic acids and hydrocarbons as well as patentability. In relation to lignocellulose enzymatic hydrolysis, it was decided to study whether *A. carbonarius* crude enzymes could be improved or enhanced towards a more efficient hydrolysis. There are many ways in which this could be achieved as can be seen in Fig. 1.3, where some of the possible routes of improvement are shown along with the strategies studied during this thesis.

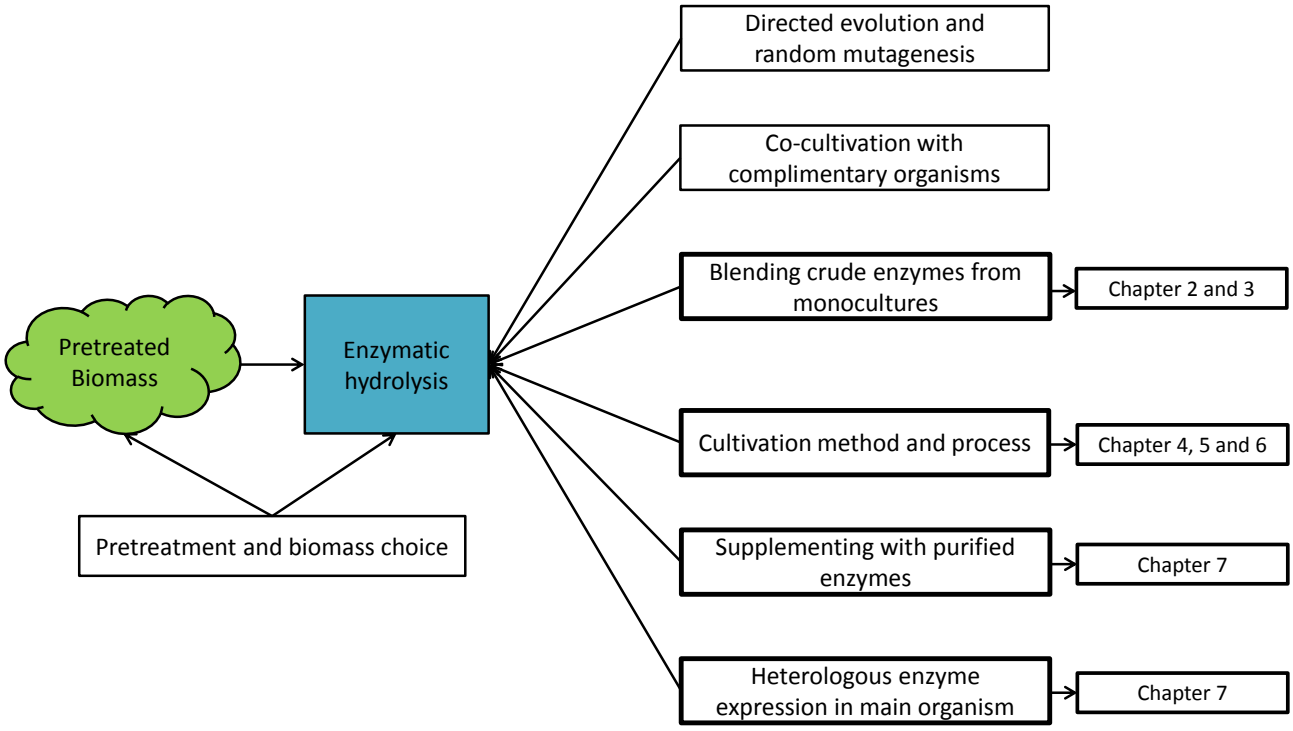


**Figure 1.3** Strategies for improving lignocellulose, the boxes framed in bold are the strategies analyzed in this thesis.

All strategies applied to improve *A. carbonarius* hydrolysis potential are shown with a bold border in Fig. 1.3. The blending of crude enzymes is evaluated in chapters 2 and 3, cultivation conditions are touched upon in chapters 4, 5 and 6 whereas the final chapter concerns the two strategies purified enzyme supplementation and heterologous enzyme expression in *A. carbonarius*. In the following sections all seven strategies presented in Fig. 1.3 are described.

## 1.3 Pretreatment and choice of biomass

There are many ways in which the lignocellulosic biomass can be pretreated. Each method affects the final composition differently, which ultimately has an impact on the enzymatic hydrolysis. A collaboration between 5 Universities and the national renewable energy laboratory (NREL) conducted two large studies on leading pretreatment technologies applied to the same biomass, namely corn stover [36]. Identical analytical methods and the same starting batch of corn stover were applied by all participants, including identical enzyme preparations and loadings. The results are interesting due to the fact that the overall sugar yield ranged from 85 to 95 % in the first study, and from 86 to 94 % in the second study [36]. These differences have an impact on the choice of process, enzymes and fermentation organism. It is important to remember that these results are specifically for corn stover, however, similar results can be expected for other agricultural residues such as wheat or rice straws [37].

### 1.3.1 *Dilute acid hydrolysis*

Complete hydrolyis of lignocellulosic material without enzyme addition has been performed using dilute acid hydrolysis, since the beginning of the 1930's (The Scholler or Madison percolation process) [38,39]. Generally $H_2SO_4$ have been used due to lower price and fewer issues with corrosion compared to *e.g.* HCl. But the overall glucose yield rarely exceeds 55-60 % of the theoretical maximum [38,40]. Dilute acid hydrolysis has often been divided into two steps, the first where hemicellulose is hydrolyzed (170-90 °C and 0.5 to 1.2 % (w/w) acid) and the second where more harsh conditions (200-230 °C and 0.5 to 1.2 % (w/w) acid) are applied to hydrolyze cellulose [41,42]. The harsh conditions in the second step leads to formation of inhibitory products and to avoid this, enzymatic hydrolysis can be substituted for the second step.

### 1.3.2 *Steam explosion*

Steam explosion pretreatment can be performed with either an acid catalyst or without (auto-hydrolysis). In an auto-hydrolysis setup the lignocellulose is heated under high pressure to temperatures around 160-250 °C for several seconds up to 10 minutes [43]. Thereafter pressure is released and an explosive decompression takes place. The high temperatures promote formation of organic acids from acetyl groups present in the material, thereby causing the auto-hydrolysis.

The application of an acid catalyst is recognized as a way to increase the accessibility of the cellulose as well as increasing hydrolysis of hemicellulose without production of degradation products. Most commonly the lignocellulose material is infused with either $H_2SO_4$ or $SO_2$ before steam explosion. This is often performed at temperatures in the range of 175 -215 °C for 2 – 10 min [44]. It has also been found that a detoxification

step after enzymatic hydrolysis, by addition of laccases, could significantly improve yeast growth and ethanol production, due to removal of phenolic compounds [44]. In a study of acid impregnated wheat straw that was steam exploded, the resulting sugar yield after enzymatic hydrolysis reached 99.6 % [45]. Steam explosion with an acid catalyst in combination with a subsequent commercial (Celluclast 1.5L and Novozym 188, Novozymes A/S) enzyme hydrolysis can therefore be deemed the most optimal for wheat straw [45]. A similar approach of pretreatment in the form of dilute acid impregnation and steam explosion has been used to pretreat the wheat straw applied during this Ph.D. project.

### 1.3.3 *Summary of pretreatment methods*

Application of dilute acid hydrolysis, using 0.75 % $H_2SO_4$, on wheat straw have been shown to result in final sugar yields of 74 % [46]. In contrast, an alkaline pretreatment of wheat straw using $H_2O_2$ resulted in 96.6 % total sugar yield after enzymatic hydrolysis [47]. This shows that there are many ways in which the lignocellulosic material could be pretreated, however, with large differences in the final sugar yield. There are many other pretreatment methods than the ones presented here, i.e. ammonia fiber explosion, wet oxidation or biological pretreatment (see [48] for descriptions).

In this thesis the main biomass that was analyzed was wheat straw, therefore results obtained for optimized crude enzymes will be valid only towards this biomass. Furthermore if another biomass was used the crude enzymes applied in this study could be expected to have a different efficiency. This is based on the knowledge that differences in degree of polymerization and crystallinity of cellulose in batches of Miscanthus has been shown to affect enzymatic hydrolysis after pretreatment [49].

## 1.4 Directed evolution and random mutagenesis

Naturally occurring mutations, recombination and selection set the stage for evolution. Directed evolution mimics this by combining reiterative random mutagenesis and recombination with screening or selection for enzyme variants with improved properties [50–52]. In contrast to classical mutagenesis, a specific gene is targeted in directed evolution to obtain random changes, followed by specific screening of the mutants [53]. This technique could therefore be used to optimize specific genes yielding cellulolytic and hemicellulolytic enzymes with a higher affinity, lower $k_{cat}$, altered pH or temperature optimum. Recently a β-glucosidase with a 94 % increased $k_{cat}$ and a 144 fold higher thermostability was the result of gene shuffling of two β-glucosidase genes from *Thermobifida fusca* and *Paebibacillus polymxyxa* [54].

There is also a possibility for engineering and optimizing organisms towards improved cellulase and hemicellulase activity, lowered product inhibition or catabolite repression, co-fermentation of hexose and

pentose sugars or improved production of the desired product [55]. Historically the improvement of cellulase activity and removal of catabolite repression through random mutagenesis has been performed on one of the most studied cellulase producing organisms, namely *T. reesei* Rut-C30 [56]. This strain was optimized from wildtype *T. reesei* QM6a after three rounds of mutagenesis to have a 2.7 fold higher protein secretion as well as 2.8 times higher filter paper activity [56]. For consolidated bioprocessing it has also been attempted to create an "ideal" organism with all desired traits. There are two categories of CBP organism development: 1) engineering cellulase producers to produce ethanol or other desired products or 2) engineering organisms with desired product forming attributes to produce cellulases [55]. The ethanol producing yeast *Saccharomyces cerevisiae* has in several attempts been optimized with added CBH production [57,58]. However, in all cases the majority of crystalline cellulose in the feedstock could not be hydrolyzed, also co-expression of cellulases was a problem [55]. A random mutagenesis library was created for the cellulolytic *Fusarium oxysporum* and screened for increased alcohol tolerance [59]. This resulted in an alcohol tolerance of 6 % in the growth medium, compared to *S. cerevisiae* having a tolerance of 15 %.

For *A. carbonarius* random mutagenesis and directed evolution has not been performed as a means for improving lignocellulolytic enzyme production. However, the closely related *A. niger* was subjected to UV and γ-ray irradiation, which resulted in the mutant strain KK2 having a more thermostable β-glucosidase and twice the activity compared to the parent strain [60]. Another improvement of enzyme production by using a combination of UV irradiation and ethyl methyl sulfonate was performed on *A. niger* NCIM 563 resulting in mutants with 17 and 47 % higher phytase activity [61]. Advances in bioinformatics and genetic engineering have though enabled a far more rational approach to strain improvement than the classical approach via mutagenesis and screening [62]. Therefore in recent years there have been a rapid development within metabolic engineering for strain optimization [32,63], which will be further described in section 1.9.

### 1.5 Co-cultivation with complimentary organisms

It is expected that co-cultivation of filamentous fungi may circumvent weaknesses of one strain due to supplementation of particular enzymes from the other strain(s) and may thereby enhance lignocellulose hydrolysis [64]. It can also be applied for consolidated bioprocessing, where one organism may contribute with the necessary enzymes for conversion of lignocellulose and the other may be optimal for producing the desired product. Co-cultivation of *A. niger* with *S. cerevisiae* have resulted in an improved ethanol tolerance due to the presence of the filamentous mycelium [65].

Co-cultivation of a cellulase producing organism with a β-glucosidase producing one, would result in a complimentary mixture of crude enzymes. This has indeed been shown for *T. reesei* Rut-C30 cultivated with the good β-glucosidase producer *A. niger* [66,67]. Another study co-cultivated *Aspergillus phoenicus* and *T. reesei* Rut-C30 thereby improving hydrolysis of cellulose in manure compared to single cultures [68]. Also a study where *A. niger* and *A. oryzae* were co-cultivated with different fungi, found that both had increased activities when cultivated with *Phanerochaete chrysosporium*. Highest β-glucosidase, α-cellobiohydrolase, β-galactosidase and laccase were obtained for cultivation of *A. oryzae* with *P. chrysosporium*, and highest β-xylosidase was obtained for *A. niger* and *P. chrysosporium* [69]. It has also been shown to be an efficient approach for stimulating lignin-degrading enzyme production *e.g.* a significant increased laccase and manganese peroxidase production was found for a mixed culture of *Pleurotus ostreatus* and *Ceriporiopsis subvermispora* [70].

The ratio between the organisms applied in co-cultivation may affect the enzyme production, as indicated by a study where highest enzyme production was apparent for *P. ostreatus* inoculated with *Penicillium oxalicum* after a delay of 144 h [71]. This is furthermore dependent on each of the fungal strains growth rate as well as pathogenicity towards each other, as can be seen from Fig. 1.4, where *A. carbonarius* is out-competed in co-cultivation with fungi that grew much faster or produced high levels of fungicides.

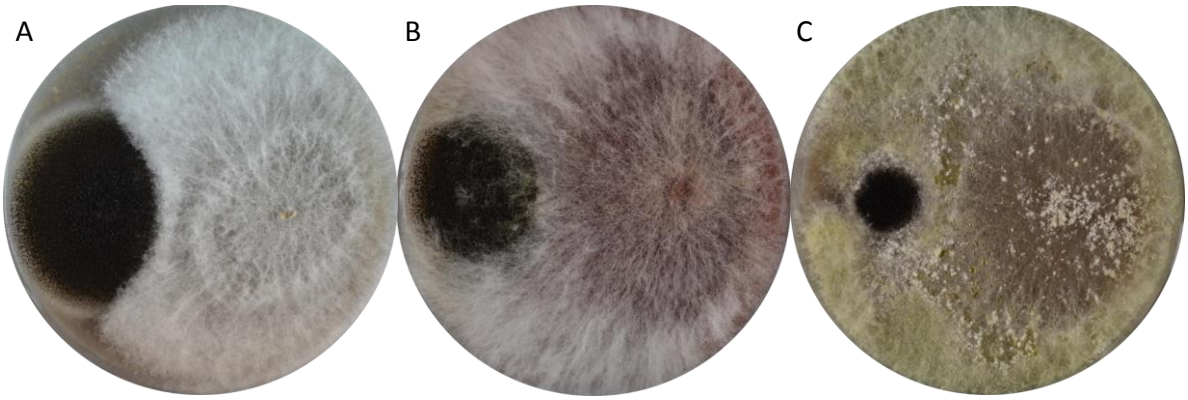


**Figure 1.4** *A. carbonarius* inoculated at the same starting time and equidistant to the co-cultivated fungi A) *Fusarium equiseti*, B) *Fusarium culmorum* and C) *Trichoderma harzianum* on wheat bran medium for 7 days.

In addition to co-cultivation, the enzyme production of a fungal strain may be optimized through addition of cell free extracts such as metabolites. This was shown for *Lentinula edodes* laccase production, which increased 15 fold relative to untreated cultures, after a shift to a medium containing cell-free liquid from *Trichoderma* sp. [72]. Another example of basidiomycetes co-cultivation resulting in higher cellulolytic activities is reported for the mixture of *Sistotrema brinkmanii* and *Agaricus arvensis* [73]. In that study an activity of 1.61 FPU/mL was obtained from the mixed culture compared to 0.3 or 0.5 FPU/mL for *S.*

*brinkmannii* or *A. arvensis* monoculture respectively. They also showed that an increased ethanol production could be obtained by co-cultivation of *S. cerevisiae* and *Pichia stipitis* which was 23 and 38 % more than their respective monocultures [73].

In relation to *A. carbonarius* there are only a limited amount of co-cultivation studies reported [28], which shows that there is a high potential of discovering improved enzymatic hydrolysis through novel co-cultivations.

### 1.6 Blending crude enzymes from monocultures

In line with co-cultivation is the separate cultivation of two or more fungi with concomitant blending/mixing of the resulting crude enzymes. This blending of two crude enzyme extracts from monocultures have been shown to be equally efficient as extracts obtained from co-cultivation in terms of hydrolysis [28]. In separate cultivation there is no risk of detrimental interaction between the strains that are producing the enzymes, as there can be in co-cultivation where strain compatibility is essential [74]. However, for large scale production two separate on-site production fermenters will increase the capital costs, therefore the gain in enzyme activity and productivity should be that much higher for it to be a feasible strategy.

Crude enzyme extracts from three white rot fungi have been analyzed for in vitro degradability of corn cob, by measuring loss of cell wall components after incubation with blends of the crude enzymes. The optimal degradation was observed when the crude enzymes of *Pleurotus florida*, *Pleurotus sajor-caju* and *Pleurotus eryngii* were mixed in a 1:1:1 ratio [74]. A study of seven separately cultivated *Penicillium* sp. and *Trichoderma* sp. showed that the complementation of *A. niger* crude enzymes containing primarily β-glucosidase and xylanase increased hydrolysis of various pretreated softwoods [75]. This effect presumably stems from alleviation of end product inhibition by cellobiose and from increased accessibility due to degradation of xyloglucan.

Therefore blending of monocultures with the crude enzymes of *A. carbonarius* appears to be an attractive strategy for improving the hydrolysis of wheat straw. This approach was followed and the outcome is presented in chapter 2.

### 1.7 Cultivation conditions

Yet another strategy for improving the efficiency of hydrolysis is by optimizing the conditions under which the enzymes are produced, to increase the yield or change the types of enzymes or ratios that are produced. There are several parameters that can be altered and optimized with regards to cultivation

including temperature, time, substrate (carbon + nitrogen) and medium composition as well as choice of bioreactor.

Especially substrate can affect the enzyme production as there are many genes that are either induced or repressed by specific carbohydrates. In *A. aculeatus* the genes *cbhI* (cellobiohydrolase), *cmc2* (endoglucanase) and *xynIa* (endo-1,4-β-xylanase) are normally induced by cellobiose and cellulose [76]. Cellulose, lactose and β-1,2-di-glucoside sophorose have been found to be optimal inducers of cellulase production in *T. reesei*, whereas xylanases are induced by L-arabinose and D-xylose [77,78]. A screening of 23 different substrates in combination with the same nitrogen source ($NaNO_3$) revealed that β-glucosidase production in *A. saccharolyticus* was induced by xylose, xylan, wheat bran and corn stover [79]. Optimizing the medium composition for cellulase production by *T. reesei* Rut-C30 resulted in 5.02 FPU/mL compared to 1.4 - 2.3 FPU/mL for the three other media analyzed [80]. The optimal medium contained a mixture of cellulose, yeast extract and additional glucose and $(NH_4)_2SO_4$, whereas the other media applied glucose + corn steep liquor, cellulose + yeast extract + proteose peptone or glucose + yeast nitrogen base + carboxymethyl cellulose (CMC) [80]. Cellulolytic enzyme production by *A. niger* HN-1 was statistically optimized resulting in 3- and 2-fold higher FP activity and BG respectively, and the achieved crude enzymes could hydrolyze rice straw glucan to glucose with 84 % efficiency [81].

Cultivation time has an impact on the enzyme production, as it was observed for *T. reesei* CAZyme gene expression, which changed as the cultivation proceeded [82]. Furthermore the cultivation temperature may affect the growth rate and the productivity of cellulases. In shake flasks cultivations it was shown to have a large impact on the EG and BG production by *A. niger* [83]. Finally it has been proposed that a solid state fermentation (SSF) system for production of enzymes can result in higher titers and less product degradation, since this system resembles the natural environment of most Ascomycetes [84]. While the submerged fermentation (SmF) system has the major advantage of being scalable and a more defined process where pH, temperature, oxygen and nutrients can be controlled during the cultivation [18].

By altering medium compositions and varying time as well as temperature it is possible to achieve a higher enzyme production, which ultimately results in improvement of crude enzyme hydrolysis of lignocellulose. The effect of applying either SSF or SmF on cellulase production is reviewed in chapter 4. Optimizing cultivation conditions for *A. carbonarius* by applying cheap waste streams as media was analyzed during this thesis and the results are described in chapter 5. Furthermore results for applying two methods for determining growth of filamentous fungi in SSF, are described in chapter 6.

## 1.8 Supplementing with purified enzymes

The knowledge of individual fungal secretomes can facilitate the design of optimal enzyme compositions for lignocellulose degradation. Especially if the fungal strain has been cultivated on different lignocellulosic sources since this will show the various strategies for lignocellulose degradation, and what may be missing in regards to the specific lignocellulosic biomass. Knowing the secretome, it becomes possible to supplement with individual enzyme activities that may be missing or that may facilitate a boosting effect. It is furthermore of importance to find a balanced ratio between the applied enzymes, as this can optimize the amount of enzyme needed thereby lowering costs without sacrificing hydrolysis efficiency [85].

An enhanced corn stover hydrolysis has been shown by supplementing a commercial cellulase blend (Celluclast 1.5 L, Novozymes) with a xylanase blend (Multifect Xylanase, Genencor) proving synergism between xylanases and cellulases [86]. For the xylanase blends it has also been shown to be of importance that arabinanase activity is present since there is a synergistic interaction. A purified endo-acting arabinanase from *P. chrysosporium* was supplemented to xylanase, and resulted in a 9.3 % higher release of sugars from barley straw, compared to single enzyme applications [87]. Also supplementation of an endo-arabinanase to the commercial Accellerase 1500 (Genencor) resulted in a 12.5 % increased glucose release from sugarcane bagasse [88]. The synergistic effect of supplementing with an endo-arabinanase was corroborated by a study where addition of pectinase and endo-arabinanase to crude enzymes of *T. harzianum* resulted in 116 % higher glucose release from sugarcane bagasse [89]. In this study they made use of secretome knowledge for *T. harzianum*, which showed that there was no production of glycosyl hydrolase (GH) families associated with arabinan, arabinogalactan or pectin degradation, which indicated the need for endo-arabinanase and pectinase [89]. Furthermore, the addition of acetyl xylan esterase can enhance enzymatic hydrolysis of wheat straw, when supplied to cellulases and xylanases, resulting in 8 % higher amount of reducing sugar release [90].

Swollenins are a fungal derived class of enzymes that can disrupt the cellulose structure, hence it would be beneficial to supplement these to an enzyme blend for lignocellulose hydrolysis [91]. A swollenin from *P. oxalicum* was purified and supplemented to cellulases in hydrolysis of crystalline avicel, resulting in a synergistic 50 % increased glucose release [92].

Recently it was shown that the addition of a lytic polysaccharide monooxygenase (LPMO), classified as an auxiliary activity family 9 (AA9) within the CAZy database (previously known as GH61), could lower the total amount of protein required by two-fold for hydrolysis of pretreated corn stover [93]. This specific LPMO

originated from *Thermoascus aurantiacus* and it has been used as a supplement for *A. carbonarius* crude enzymes, the results of which are described in chapter 7.

## 1.9 Metabolic engineering for heterologous enzyme production

The metabolic engineering approach for improving fungal strains to increase cellulase production or alter the composition of their crude enzyme blends is seemingly without limitations. The wealth of omics data for *Aspergillus* spp. provides an ideal basis for modelling and engineering strains for better productivity and improved rheological performance [63]. Metabolic engineering is not only useful for improving the cellulase production or making optimized crude enzyme blends, it also paves the way for utilizing novel substrates and improving production of products. *Aspergillus oryzae* which is the exclusive producer of kojic acid, normally has a poor cellulase production, but in a recent study it was engineered to overexpress kojic acid expression factor *kojT* and three cellulases. The resulting strain was able to produce kojic acid when grown only on cellulose. Although the yield was low, it proved the concept that *A. oryzae* could be trimmed for production of a valuable product on second generation feedstocks [94]. Attempts have also been made for shifting the high production of citric acid to itaconic acid in *A. niger* [95]. With projects like the 1000 Fungal Genomes Project or the Aspergillus Whole-Genus Sequencing project there are a rapid increase of information regarding non-model fungal organisms (http://genome.jgi.doe.gov/). Despite the great amount of information the fact that there are scarce tools for genetic manipulation of most fungi, greatly hampers the exploitation. Recently, however, a new method for genetic engineering of fungi was developed based on the archaeal immune mechanism CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9. The method allows for simple RNA guided gene targeting and was applied to six Aspergilli including generation of a strain where iterative gene targeting was possible [96]. It was also proven to be an efficient method for genome editing in *T. reesei* [97]. The CRISPR-Cas9 system significantly improves the possibilities of genetically engineering non-model fungal strains to grow on lignocellulosic waste and to overproduce cellulases. The strategies for improving cellulase production and thereby growth on lignocellulose includes overexpression of transcription factors, cellulases, xylanases, accessory enzymes, promoter selection, modification of signal transduction pathways and protein secretion pathways, alleviating carbon catabolite repression and deleting extracellular proteases [63,78,98]. Some examples of these strategies are detailed below.

### 1.9.1 *Transcription factors*

ClbR is a transcriptional activator controlling expression of cellulase genes in response to avicel and cellobiose for *Aspergillus aculeatus* [99], and homologs are known in *A. niger*, *Neurospora crassa* and

*Fusarium graminearum* [100]. Overexpression of ClbR in *A. aculeatus* resulted in a 9- and 2.3-fold higher β-1,4-xylanase and β-1,4-endoglucanase activity, respectively, but activities of β-glucosidase, carboxymethyl cellulase and avicelase were unchanged [99]. The xylan dependent transcription activator XlnR have also been overexpressed in *A. oryzae* and *A. niger* leading to increased expression of both xylanase and cellulase genes [101,102]. Furthermore the glucose repressor gene *cre1* of *T. reesei* is responsible for repression of hemicellulase and cellulase expression in glucose containing media [56]. However, truncation of this gene also resulted in higher cellulase and hemicellulase of *T. reesei* QM6a in hydrolase inducing medium compared to wildtype [103]. This indicates that the *cre1* gene may also be a target for improving cellulase and hemicellulase production of strains to be grown in lignocellulosic media.

### 1.9.2 *Expression of additional lignocellulolytic enzymes*

Optimized utilization of lignocellulose waste can be enabled by expression of additional lignocellulolytic enzymes in different fungal strains. An example is the expression of the Cel7B gene (encoding an EG) in *A. niger*, the mutant strain was able to grow well on spent hydrolysate (stillage) of sugarcane bagasse and spruce wood producing a biomass dry weight of 9.8 g/L compared to the negative control having 6.8 g/L [104]. Also the EG activity of the mutant was higher in the spent hydrolysate medium (2200 nkat/mL) than in a standard glucose medium (480 nkat/mL) [104]. This strain could therefore be ideally used for on-site production of enzymes in a biorefinery setting. Despite this, there may be disadvantages of transferring enzyme genes into expression hosts such as *A. niger*. It has been shown that the production of Cel7A in *A. niger* resulted in a CBH with lower specific activity towards cellulose compared to the same enzyme produced in *T. reesei,* which the CBH originates from [105]. The cause of it was the alternative protein processing in *A. niger* which led to a more glycosylated CBH. It was also shown that by enzymatically removing glycosylation of the CBH the activity returned to that of the CBH produced in *T. reesei* [105]. It has also been attempted to create a cellulolytic *S. cerevisiae* strain in order to achieve a candidate for CBP of cellulose to ethanol, in this regard four CBH genes were successfully expressed but titer levels were relatively low [106].

Other enzymes that may be expressed in combination with the CBH and EG are enzymes belonging to the auxiliary activities (AA) class as defined by the CAZy database. Especially the AA9 family is of interest, since this class consists of lytic polysaccharide monooxygenases (previously known as GH61) for which significant boosting effects of lignocellulose hydrolysis have been observed [93]. It is also a component in the commercial cellulase preparation Cellic CTec2, and one of the reasons for the markedly improved efficiency compared to its predecessor [107]. In another attempt at making *S. cerevisiae* suitable for a CBP process of converting cellulose to ethanol, a mini-cellulosome containing LPMO, CDH, CBH, EG and BG was

heterologously expressed on the yeast cell surface [108]. The resulting strain was able to grow solely on phosphoric acid swollen cellulose (PASC) and produced an ethanol titer of 2.7 g/L. Furthermore, the hydrolysis of PASC increased by the application of LPMO and CDH in the cellulosome compared to a cellulosome of only CBH, EG and BG [108].

There are three classes of LPMO's determined by their preferred site of oxidation. Type 1 and 2 oxidize at C1 and C4 carbon atoms of glucose, while type 3 oxidize at both positions [109]. A *T. aurantiacus* LPMO (GH61A) has been proposed as a type 3 [110] with possible oxidation of C6 and for a *Podospora anserina* LPMO (*Pa*GH61B) only C6 oxidation has been suggested [111]. The same *T. aurantiacus* LPMO was heterologously expressed in *T. reesei* whereby the overall protein loading for reaching 91 % hydrolysis efficiency was reduced from 8 mg protein/g cellulose to 4.2 mg protein/g cellulose corresponding to a 1.9-fold decrease in protein loading [93]. Both *T. aurantiacus* and *P. anserina* LPMO's were applied as single enzyme supplements for the crude enzymes of *A. carbonarius*. The LPMO from *P. anserina* was also heterologously expressed in *A. carbonarius* to further study its effect on the resulting crude enzymes. Results of these experiments are described in chapter 7.

## 1.10 References


[1] Jäger G, Büchs J. Biocatalytic conversion of lignocellulose to platform chemicals. Biotechnol J 2012;7:1122–36.

[2] Limayem A, Ricke SC. Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Prog Energy Combust Sci 2012;38:449–67.

[3] López-Abelairas M, Lu-Chau TA, Lema JM. Enhanced saccharification of biologically pretreated wheat straw for ethanol production. Appl Biochem Biotechnol 2013;169:1147–59.

[4] Sánchez C. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 2009;27:185–94.

[5] Van Dyk JS, Pletschke BI. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes--factors affecting enzymes, conversion and synergy. Biotechnol Adv 2012;30:1458–80.

[6] Thygesen A, Thomsen AB, Schmidt AS, Jørgensen H, Ahring BK, Olsson L. Production of cellulose and hemicellulose-degrading enzymes by filamentous fungi cultivated on wet-oxidised wheat straw. Enzyme Microb Technol 2003;32:606–15.

[7] Rubin EM. Genomics of cellulosic biofuels. Nature 2008;454:841–5.

[8] Werpy T, Petersen G. Top Value Added Chemicals from Biomass. 2004.

[9] Bond JQ, Upadhye AA, Olcay H, Tompsett GA, Jae J, Xing R, et al. Production of renewable jet fuel range alkanes and commodity chemicals from integrated catalytic processing of biomass. Energy Environ Sci 2014;7:1500–23.

[10] Ekman A, Wallberg O, Joelsson E, Börjesson P. Possibilities for sustainable biorefineries based on agricultural residues – A case study of potential straw-based ethanol production in Sweden. Appl Energy 2013;102:299–308.

[11] Sørensen A, Lübeck M, Lübeck P, Ahring B. Fungal beta-glucosidases: A bottleneck in industrial use of lignocellulosic materials. Biomolecules 2013;3:612–31.

[12] Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol Mol Biol Rev 2002;66:506–77.

[13] Lau MW, Bals BD, Chundawat SPS, Jin M, Gunawan C, Balan V, et al. An integrated paradigm for cellulosic biorefineries: utilization of lignocellulosic biomass as self-sufficient feedstocks for fuel, food precursors and saccharolytic enzyme production. Energy Environ Sci 2012;5:7100.

[14] Himmel ME, Ding S, Johnson DK, Adney WS, Nimlos MR, Brady JW, et al. Biomass recalcitrance: Engineering plants and enzymes for biofuels production. Science 2007;315:804–7.

[15] Haltrich D, Nidetzky B, Kulbe KD, Zupancic S. Production of fungal xylanases. Bioressource Technol 1997;58:137–61.

[16] Hooge DM, Pierce JL, McBride KW, Rigolin PJ. Meta-analysis of broiler chicken trials using diets with or without allzyme SSF enzyme complex. Int J Poult Sci 2010;9:819–23.

[17] Prévot V, Lopez M, Copinet E, Duchiron F. Comparative performance of commercial and laboratory enzymatic complexes from submerged or solid-state fermentation in lignocellulosic biomass hydrolysis. Bioresour Technol 2013;129:690–3.

[18] Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme Microb Technol 2010;46:541–9.

[19] Hansen GH, Lübeck M, Frisvad JC, Lübeck PS, Andersen B. Production of cellulolytic enzymes from ascomycetes: Comparison of solid state and submerged fermentation. Process Biochem 2015;50:1327–41.

[20] Dashtban M, Schraft H, Qin W. Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. Int J Biol Sci 2009;5:578–95.

[21] Juturu V, Wu JC. Microbial xylanases: engineering, production and industrial applications. Biotechnol Adv 2012;30:1219–27.

[22] Resch MG, Donohoe BS, Baker JO, Decker SR, Bayer EA, Beckham GT, et al. Fungal cellulases and complexed cellulosomal enzymes exhibit synergistic mechanisms in cellulose deconstruction. Energy Environ Sci 2013;6:1858.

[23] Chiaramonti D, Prussi M, Ferrero S, Oriani L, Ottonello P, Torre P, et al. Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method. Biomass and Bioenergy 2012;46:25–35.

[24] Gottschalk LMF, Oliveira RA, Bon EPDS. Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. Biochem Eng J 2010;51:72–8.

[25] Sørensen A, Teller PJ, Lübeck PS, Ahring BK. Onsite enzyme production during bioethanol production from biomass: screening for suitable fungal strains. Appl Biochem Biotechnol 2011;164:1058–70.

[26] Sukumaran RK, Singhania RR, Mathew GM, Pandey A. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. Renew Energy 2009;34:421–4.

[27] Rana V, Eckard AD, Teller P, Ahring BK. On-site enzymes produced from *Trichoderma reesei* RUT-C30 and *Aspergillus saccharolyticus* for hydrolysis of wet exploded corn stover and loblolly pine. Bioresour Technol 2014;154:282–9.

[28] Kolasa M, Ahring BK, Lübeck PS, Lübeck M. Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. Bioresour Technol 2014;169:143–8.

[29] Perrone G, Mule G, Susca A, Battilani P, Pietri A, Logrieco A. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. Society 2006;72:680–5.

[30] Weyda I, Lübeck M, Ahring BK, Lübeck PS. Point mutation of the xylose reductase (XR) gene reduces xylitol accumulation and increases citric acid production in *Aspergillus carbonarius*. J Ind Microbiol Biotechnol 2014;41:733–9.

[31] Yang L, Lübeck M, Lübeck PS. Deletion of glucose oxidase changes the pattern of organic acid production in *Aspergillus carbonarius*. AMB Express 2014;4:54. doi:10.1186/s13568-014-0054-7.

[32] Andersen MR, Nielsen J. Current status of systems biology in *Aspergilli*. Fungal Genet Biol 2009;46 Suppl 1:S180–90.

[33] Sinha M, Sørensen A, Ahamed A, Ahring BK. Production of hydrocarbons by *Aspergillus carbonarius* ITEM 5010. Fungal Biol 2015;119:274–82.

[34] Kiss T, Kiss L. Purification and characterization of an extracellular beta-D-xylosidase from *Aspergillus carbonarius*. World J Microbiol Biotechnol 2000;16:465–70.

[35] Meijer M, Houbraken JAMP, Dalhuijsen S, Samson RA, de Vries RP. Growth and hydrolase profiles can be used as characteristics to distinguish *Aspergillus niger* and other black *aspergilli*. Stud Mycol 2011;69:19–30.

[36] Elander RT, Dale BE, Holtzapple M, Ladisch MR, Lee YY, Mitchinson C, et al. Summary of findings from the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI): Corn stover pretreatment. Cellulose 2009;16:649–59.

[37] Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresour Technol 2005;96:2026–32.

[38] Parisi F. Advances in lignocellulosics hydrolysis and in the utilization of the hydrolyzates. Lignocellul. Mater. SE - 2, vol. 38, Springer Berlin Heidelberg; 1989, p. 53–87.

[39] Jones JL, Semrau KT. Wood hydrolysis for ethanol production — previous experience and the economics of selected processes. Biomass 1984;5:109–35.

[40] Lee YY, Iyer P, Torget RW. Dilute-acid hydrolysis of lignocellulosic biomass In: Tsao GT, Brainard AP, Bungay HR, Cao NJ, Cen P, Chen Z, et al., editors. Recent Prog. Bioconversion Lignocellul. SE - 5, vol. 65, Springer Berlin Heidelberg; 1999, p. 93–115. doi:10.1007/3-540-49194-5_5.

[41] Kim KH, Tucker MP, Nguyen QA. Effects of pressing lignocellulosic biomass on sugar yield in two-stage dilute-acid hydrolysis process. Biotechnol Prog 2002;18:489–94.

[42] Neureiter M, Danner H, Thomasser C, Saidi B, Braun R. Dilute-acid hydrolysis of sugarcane bagasse at varying conditions. Appl Biochem Biotechnol 2002;98-100:49–58.

[43] Ballesteros I, Negro MJ, Oliva JM, Cabañas A, Manzanares P, Ballesteros M. Ethanol production from steam-explosion pretreated wheat straw. Appl Biochem Biotechnol 2006;129-132:496–508.

[44] Jurado M, Prieto A, Martínez-Alcalá Á, Martínez ÁT, Martínez MJ. Laccase detoxification of steam-exploded wheat straw for second generation bioethanol. Bioresour Technol 2009;100:6378–84.

[45] Linde M, Jakobsson EL, Galbe M, Zacchi G. Steam pretreatment of dilute H2SO4-impregnated wheat straw and SSF with low yeast and enzyme loadings for bioethanol production. Biomass and Bioenergy 2008;32:326–32.

[46] Saha BC, Iten LB, Cotta MA, Wu YV. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochem 2005;40:3693–700.

[47] Saha BC, Cotta MA. Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw. Biotechnol Prog 2006;22:449–53.

[48] Talebnia F, Karakashev D, Angelidaki I. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. Bioresour Technol 2010;101:4744–53.

[49] Zhang W, Yi Z, Huang J, Li F, Hao B, Li M, et al. Three lignocellulose features that distinctively affect biomass enzymatic digestibility under NaOH and H2SO4 pretreatments in Miscanthus. Bioresour Technol 2013;130:30–7.

[50] Cherry JR, Fidantsef AL. Directed evolution of industrial enzymes: An update. Curr Opin Biotechnol 2003;14:438–43.

[51] Tobin MB, Gustafsson C, Huisman GW. Directed evolution: The “rational” basis for “irrational” design. Curr Opin Struct Biol 2000;10:421–7.

[52] Chirumamilla RR, Muralidhar R, Marchant R, Nigam P. Improving the quality of industrially important enzymes by directed evolution. Mol Cell Biochem 2001;224:159–68.

[53] Antikainen NM, Martin SF. Altering protein specificity: Techniques and applications. Bioorganic Med Chem 2005;13:2701–16.

[54] Pei XQ, Yi ZL, Tang CG, Wu ZL. Three amino acid changes contribute markedly to the thermostability of β-glucosidase BglC from *Thermobifida fusca*. Bioresour Technol 2011;102:3337–42.

[55] Den Haan R, van Rensburg E, Rose SH, Görgens JF, van Zyl WH. Progress and challenges in the engineering of non-cellulolytic microorganisms for consolidated bioprocessing. Curr Opin Biotechnol 2015;33:32–8.

[56] Peterson R, Nevalainen H. *Trichoderma reesei* RUT-C30--thirty years of strain improvement. Microbiology 2012;158:58–68.

[57] Ilmén M, den Haan R, Brevnova E, McBride J, Wiswall E, Froehlich A, et al. High level secretion of cellobiohydrolases by *Saccharomyces cerevisiae*. Biotechnol Biofuels 2011;4:30.

[58] Mcbride J, Brevnova E, Mellon M, Application F, Data P, Uusitalo J, et al. Yeast expressing cellulases for simultaneous saccharification and fermentation using cellulose. US. Patent. US 2012/0129229 A1, 2012.

[59] Hennessy RC, Doohan F, Mullins E. Generating phenotypic diversity in a fungal biocatalyst to investigate alcohol stress tolerance encountered during microbial cellulosic biofuel production. PLoS One 2013;8:1–13.

[60] Kang SW, Ko EH, Lee JS, Kim SW. Over-production of β-glucosidase by *Aspergillus niger* mutant from lignocellulosic biomass. Biotechnol Lett 1999;21:647–50.

[61] Shah P, Bhavsar K, Soni SK, Khire JM. Strain improvement and up scaling of phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. J Ind Microbiol Biotechnol 2009;36:373–80.

[62] Nielsen J. Metabolic engineering. Appl Microbiol Biotechnol 2001;55:263–83.

[63] Meyer V, Fiedler M, Nitsche B, King R. The cell factory aspergillus enters the big data era: Opportunities and challenges for optimising product formation. Adv Biochem Eng Biotechnol 2015;149:91–132.

[64] Adav SS, Ravindran A, Cheow ESH, Sze SK. Quantitative proteomic analysis of secretome of microbial consortium during saw dust utilization. J Proteomics 2012;75:5590–603.

[65] Ohta K, Hamada S, Nakamura T. Production of high concentrations of ethanol from inulin by simultaneous saccharification and fermentation using *Aspergillus niger* and *Saccharomyces cerevisiae*. Appl Environ Microbiol 1993;59:729–33.

[66] Ahamed A, Vermette P. Enhanced enzyme production from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* LMA grown as fed batch in a stirred tank bioreactor. Biochem Eng J 2008;42:41–6.

[67] Fang H, Zhao C, Song XY. Optimization of enzymatic hydrolysis of steam-exploded corn stover by two approaches: Response surface methodology or using cellulase from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* NL02. Bioresour Technol 2010;101:4111–9.

[68] Wen Z, Liao W, Chen S. Production of cellulase/β-glucosidase by the mixed fungi culture *Trichoderma reesei* and *Aspergillus phoenicis* on dairy manure. Process Biochem 2005;40:3087–94.

[69] Hu HL, van den Brink J, Gruben BS, Wösten HAB, Gu JD, de Vries RP. Improved enzyme production by co-cultivation of *Aspergillus niger* and *Aspergillus oryzae* and with other fungi. Int Biodeterior Biodegrad 2011;65:248–52.

[70] Chi Y, Hatakka A, Maijala P. Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin-degrading enzymes? Int Biodeterior Biodegrad 2007;59:32–9.

[71] Dwivedi P, Vivekanand V, Pareek N, Sharma A, Singh RP. Co-cultivation of mutant *Penicillium oxalicum* SAU E-3.510 and *Pleurotus ostreatus* for simultaneous biosynthesis of xylanase and laccase under solid-state fermentation. N Biotechnol 2011;28:616–26.

[72] Savoie JM, Mata G, Billette C. Extracellular laccase production during hyphal interactions between *Trichoderma* sp. and Shiitake, *Lentinula edodes*. Appl Microbiol Biotechnol 1998;49:589–93.

[73] Kalyani D, Lee K-M, Kim T-S, Li J, Dhiman SS, Kang YC, et al. Microbial consortia for saccharification of woody biomass and ethanol fermentation. Fuel 2013;107:815–22.

[74] Dong YC, Wang W, Hu ZC, Fu ML, Chen QH. The synergistic effect on production of lignin-modifying enzymes through submerged co-cultivation of *Phlebia radiata*, *Dichomitus squalens* and *Ceriporiopsis subvermispora* using agricultural residues. Bioprocess Biosyst Eng 2012;35:751–60.

[75] Berlin A, Gilkes N, Kilburn D, Bura R, Markov A, Skomarovsky A, et al. Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates - Evidence for the role of accessory enzymes. Enzyme Microb Technol 2005;37:175–84.

[76] Kunitake E, Tani S, Sumitani J, Kawaguchi T. A novel transcriptional regulator, ClbR, controls the cellobiose- and cellulose-responsive induction of cellulase and xylanase genes regulated by two distinct signaling pathways in *Aspergillus aculeatus*. Appl Microbiol Biotechnol 2013;97:2017–28.

[77] Herold S, Bischof R, Metz B, Seiboth B, Kubicek CP. Xylanase gene transcription in *Trichoderma reesei* is triggered by different inducers representing different hemicellulosic pentose polymers. Eukaryot Cell 2013;12:390–8.

[78] Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B. Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol Biofuels 2009;2:19.

[79] Sørensen A, Andersen JJ, Ahring BK, Teller PJ, Lübeck M. Screening of carbon sources for beta-glucosidase production by *Aspergillus saccharolyticus*. Int Biodeterior Biodegradation 2014;93:78–83.

[80] Ahamed A, Vermette P. Culture-based strategies to enhance cellulase enzyme production from *Trichoderma reesei* RUT-C30 in bioreactor culture conditions. Biochem Eng J 2008;40:399–407.

[81] Sandhu SK, Oberoi HS, Babbar N, Miglani K, Chadha BS, Nanda DK. Two-stage statistical medium optimization for augmented cellulase production via solid-state fermentation by newly isolated *Aspergillus niger* HN-1 and application of crude cellulase consortium in hydrolysis of rice straw. J Agric Food Chem 2013;61:12653–61.

[82] Häkkinen M, Arvas M, Oja M, Aro N, Penttilä M, Saloheimo M, et al. Re-annotation of the CAZy genes of *Trichoderma reesei* and transcription in the presence of lignocellulosic substrates. Microb Cell Fact 2012;11:134.

[83] Sohail M, Siddiqi R, Ahmad A, Khan SA. Cellulase production from *Aspergillus niger* MS82: effect of temperature and pH. N Biotechnol 2009;25:437–41.

[84] Viniegra-González G, Favela-Torres E, Aguilar CN, Rómero-Gomez SDJ, Díaz-Godínez G, Augur C. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochem Eng J 2003;13:157–67.

[85] Badhan A, Wang Y, Gruninger R, Patton D, Powlowski J, Tsang A, et al. Formulation of enzyme blends to maximize the hydrolysis of alkaline peroxide pretreated alfalfa hay and barley straw by rumen enzymes and commercial cellulases. BMC Biotechnol 2014;14:31.

[86] Hu J, Arantes V, Saddler JN. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Biotechnol Biofuels 2011;4:36.

[87] Huy N, Thiyagarajan S, Choi Y-E, Kim D-H, Park S-M. Cloning and characterization of a thermostable endo-arabinanase from *Phanerochaete chrysosporium* and its synergistic action with endo-xylanase. Bioprocess Biosyst Eng 2013;36:677–85.

[88] Machado CB, Citadini AP, Goldbeck R, de Lima EA, Figueiredo FL, da Silva TM, et al. Increased biomass saccharification by supplementation of a commercial enzyme cocktail with endo-arabinanase from *Bacillus licheniformis*. Biotechnol Lett 2015;37:1455–62.

[89] Delabona PDS, Cota J, Hoffmam ZB, Paixão DAA, Farinas CS, Cairo JPLF, et al. Understanding the cellulolytic system of *Trichoderma harzianum* P49P11 and enhancing saccharification of pretreated sugarcane bagasse by supplementation with pectinase and α-l-arabinofuranosidase. Bioresour Technol 2013;131:500–7.

[90] Zhang J, Siika-aho M, Tenkanen M, Viikari L. The role of acetyl xylan esterase in the solubilization of xylan and enzymatic hydrolysis of wheat straw and giant reed. Biotechnol Biofuels 2011;4:60.

[91] Jäger G, Girfoglio M, Dollo F, Rinaldi R, Bongard H, Commandeur U, et al. How recombinant swollenin from *Kluyveromyces lactis* affects cellulosic substrates and accelerates their hydrolysis. Biotechnol Biofuels 2011;4:33.

[92] Kang K, Wang S, Lai G, Liu G, Xing M. Characterization of a novel swollenin from *Penicillium oxalicum* in facilitating enzymatic saccharification of cellulose. BMC Biotechnol 2013;13:42.

[93] Harris PV, Welner D, McFarland KC, Re E, Navarro Poulsen J-C, Brown K, et al. Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry 2010;49:3305–16.

[94] Yamada R, Yoshie T, Wakai S, Asai-Nakashima N, Okazaki F, Ogino C, et al. *Aspergillus oryzae*-based cell factory for direct kojic acid production from cellulose. Microb Cell Fact 2014;13:71.

[95] Van der Straat L, Vernooij M, Lammers M, van den Berg W, Schonewille T, Cordewener J, et al. Expression of the *Aspergillus terreus* itaconic acid biosynthesis cluster in *Aspergillus niger*. Microb Cell Fact 2014;13:11.

[96] Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH. A CRISPR-Cas9 System for Genetic Engineering of Filamentous Fungi. PLoS One 2015;10:e0133085.

[97] Liu R, Chen L, Jiang Y, Zhou Z, Zou G. Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. Cell Discov 2015;1:15007.

[98] Ward OP. Production of recombinant proteins by filamentous fungi. Biotechnol Adv 2012;30:1119–39.

[99] Kunitake E, Kawamura A, Tani S, Takenaka S, Ogasawara W, Sumitani J, et al. Effects of clbR overexpression on enzyme production in *Aspergillus aculeatus* vary depending on the cellulosic biomass-degrading enzyme species. Biosci Biotechnol Biochem 2014;79:488–95.

[100] Steindorff AS, Persinoti GF, Monteiro VN, Silva RN. Fungal Metabolic Diversity. In: Gupta VK, Mach RL, Sreenivasaprasad S, editors. Fungal Biomol. Sources, Appl. Recent Dev. 1st ed., John Wiley & Sons, Ltd.; 2015, p. 246–7.

[101] Van Peij NNME, Gielkens MMC, de Vries RP, Visser J, de Graaff LH. The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. Appl Environ Microbiol 1998;64:3615–9.

[102] Marui J, Kitamoto N, Kato M, Kobayashi T, Tsukagoshi N. Transcriptional activator, AoXlnR, mediates cellulose-inductive expression of the xylanolytic and cellulolytic genes in *Aspergillus oryzae*. FEBS Lett 2002;528:279–82.

[103] Nakari-Setälä T, Paloheimo M, Kallio J, Vehmaanperä J, Penttilä M, Saloheimo M. Genetic modification of carbon catabolite repression in *Trichoderma reesei* for improved protein production. Appl Environ Microbiol 2009;75:4853–60.

[104] Alriksson B, Rose SH, Van Zyl WH, Sjöde A, Nilvebrant NO, Jönsson LJ. Cellulase production from spent lignocellulose hydrolysates by recombinant *Aspergillus niger*. Appl Environ Microbiol 2009;75:2366–74.

[105] Jeoh T, Michener W, Himmel ME, Decker SR, Adney WS. Implications of cellobiohydrolase glycosylation for use in biomass conversion. Biotechnol Biofuels 2008;1:10.

[106] Den Haan R, Mcbride JE, Grange DC La, Lynd LR, Van Zyl WH. Functional expression of cellobiohydrolases in *Saccharomyces cerevisiae* towards one-step conversion of cellulose to ethanol. Enzyme Microb Technol 2007;40:1291–9.

[107] Horn SJ, Vaaje-Kolstad G, Westereng B, Eijsink VG. Novel enzymes for the degradation of cellulose. Biotechnol Biofuels 2012;5:45.

[108] Liang Y, Si T, Ang EL, Zhao H. An engineered penta-functional minicellulosome for simultaneous saccharification and ethanol fermentation in *Saccharomyces cerevisiae*. Appl Environ Microbiol 2014;80:6677–84.

[109] Bennati-granier C, Garajova S, Champion C, Grisel S, Haon M, Zhou S, et al. Substrate specificity and regioselectivity of fungal AA9 lytic polysaccharide monooxygenases secreted by *Podospora anserina*. Biotechnol Biofuels 2015:1–14.

[110] Quinlan RJ, Sweeney MD, Leggio L Lo, Otten H, Poulsen J-CN, Johansen KS, et al. Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. Proc Natl Acad Sci U S A 2011;108:15079–84.

[111] Bey M, Zhou S, Poidevin L, Henrissat B, Coutinho PM, Berrin J-G, et al. Cello-oligosaccharide oxidation reveals differences between two lytic polysaccharide monooxygenases (family GH61) from *Podospora anserina*. Appl Environ Microbiol 2013;79:488–96.

# Chapter 2

# Screening for cellulase producers and boosters of lignocellulose hydrolysis in different environmental niches

Manuscript in preparation

This chapter mainly concerns preliminary screening experiments, presented in a manuscript form.

# Screening for cellulase producers and boosters of lignocellulose hydrolysis in different environmental niches

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*Keywords*: *Aspergillus carbonarius*; Cellulase screening; Crude enzymes; Hydrolysis synergy; Ascomycetes;

## 2.1 Abstract

For this study fungi isolated from different environmental niches, all containing lignocellulosic plant biomass, were analyzed for their enzyme activities. A high endo-1,4-β-xylanase activity was observed in crude enzymes obtained from cultivations of 16 fungal species grown on wheat bran agar, and all species except two could produce endo-glucanase activity. However, there was in general a low expression of endo-1,4-β-mannanase. The crude enzymes were also analyzed for whether they could be applied as synergistic supplements for *Aspergillus carbonarius* crude enzymes applied in wheat straw hydrolysis. There was a significant 3-fold improvement of glucose release when crude enzymes from the indoor fungal isolates *Penicillium allii*, *P. olsonii*, *P. polonicum* and *Aspergillus niger* were applied. The same was apparent for two coprophilic fungal isolates *P. vulpinum* and *P. coprophilum* as well as for two isolates from garden and park waste *Gliocladium* sp. 1 and 2. When scaling the enzyme production to shake flask cultures, the boosting effects changed to being only apparent for *Gliocladium* sp. 1, *Fusarium poae* and *F. graminearum* isolated from barley. This indicates that the boosting effect was highly dependent on the medium composition as well as conditions of cultivation (submerged or solid state). Furthermore the dosage of the crude enzymes had an influence on the observed synergy, as there was no synergy when half the enzyme loadings were applied for the crude enzymes obtained from submerged cultivations. The most promising boosting effect was seen for the *Gliocladium* sp. 1 isolate, which was also the most consistent effect. This isolate could therefore be applied as a supplementing organism in a process utilizing *A. carbonarius* as an on-site enzyme producer.

*Keywords*: *Aspergillus carbonarius*; Cellulase screening; Crude enzymes; Hydrolysis synergy; Ascomycetes;

## 2.2 Introduction

Hydrolysis of lignocellulose can nowadays be performed to a high degree of efficiency by using commercial enzyme preparations Cellic® CTec3 (Novozymes A/S), Acellerase® Trio™ (Genencor), AlternaFuel® CMAX™3 (Dyadic International Inc.) or Jtherm® (JBEI). These commercial enzyme cocktails are, however, considered expensive [1] and have a negative impact on the economic feasibility of a biorefinery conversion of lignocellulose to valuable products such as organic acids and sustainable bioenergy. There are several ways that the enzyme costs can be lowered e.g. by on-site enzyme production utilizing a waste stream from the biorefinery as substrate or by simultaneous saccharification and fermentation in which one strain hydrolyze and ferments the lignocellulose [2–4].

The on-site enzyme production strategy can be further optimized by taking advantage of co-cultivations of two or more cellulase producing strains, in order to create an overall improved crude enzyme mixture. Previously it has been shown that co-cultivation of *Trichoderma reesei* Rut-C30 (a hypercellulase producer) with *Aspergillus saccharolyticus* or *A. carbonarius* resulted in 80 % hydrolysis efficiency compared to 50 % efficiency of the *T. reesei* monoculture enzymes [5]. Other examples of co-cultivations for improvement of crude enzymes are *Penicillium oxalicum* and *Pleurotus ostreatus, Schizophylum commune* and *Mucor* sp., *A. oryzae* and *T. reesei* as well as *A. ellipticus* and *A. fumigatus* among many others [6–9]. Higher enzyme activities were obtained in all cases of co-cultivation compared to mono-cultures, indicating potential for their application as on-site enzyme producers. Blends of mono-cultures have also been proven efficient for achieving an effective hydrolysis. In the case of *T. reesei* and *A. awamori*, monocultures were produced from a wheat bran (WB) medium and crude enzymes were concentrated before being mixed in different ratios. All blends resulted in 1.6 g/L higher glucose yield (80 % efficiency) compared to monocultures of either fungi [10]. Synergy or complementarity of crude enzymes have also been evaluated for *Chrysoporthe cubensis* and *Talaromyces pinophilus* (previously *Penicillium pinophilum*), where an increase of FPase, endoglucanase and xylanase activities was found [11]. Blending of crude enzymes from monocultures can be used to screen for co-cultivation candidates, as it was shown by Kolasa et al. [5], that the hydrolysis efficiency of blending monocultures were equal to the crude enzymes obtained from co-cultivations.

In addition to enzyme production, *A. carbonarius* has recently been shown to be able to produce a series of hydrocarbons in liquid culture using lignocellulosic biomasses, such as corn stover and switch grass as carbon source [12]. *A. carbonarius* is also capable of producing organic acids from different kinds of carbon sources including glucose, xylose, sucrose and galactose [13–15], and in a wheat straw hydrolysate (unpublished data). Combining effects of lignocellulosic conversion and production in the same fungus would make it possible to develop a consolidated bioprocess. However, due to the recalcitrant nature of

lignocellulosic material, the enzymes produced by *A. carbonarius* are not efficient without supplements of other enzymes whether in the form of crude enzyme extracts from other fungi or by co-cultivation.

The aim of this study was to analyze fungal strains isolated from different niches containing lignocellulose: indoor environment (wooden building materials), barley, ruminant dung as well as lignocellulosic garden and park waste (GPW) for their potential as boosters of *A. carbonarius* lignocellulose conversion. Synergy or boosting effects were analyzed by blending crude enzymes from monocultures and applying them in wheat straw hydrolysis.

## 2.3 Methods and materials

### 2.2.1 *Isolation and identification of fungal strains*

Garden and park waste (GPW) was collected at a commercial compost pile site at Solum A/S. The GPW was relatively fresh (non-composted) and contained a mixture of all types of garden and park trimmings (branches and leaves). From this mixture branches and leaves that could be determined to have mold growth on them, were selected and streaked on two V8-juice agar (V8) plates [16] leaving the piece on the second plate. Fungi were isolated after 5-14 days incubation at 25 °C and transferred to a new V8 plate. The isolated pure cultures were identified by morphology after growing the individual strains on several media (malt extract agar (MEA), Czapek yeast extract agar (CYA), dichloran 18 % glycerol agar (DG18), creatine sucrose agar (CREA), yeast extract sucrose (YES), potato dextrose agar (PDA), spezieller nährstoffarmer agar (SNA) and V8) [16]. Fungi from the IBT collection were furthermore selected for the screening. The selected fungi were chosen based on the isolates origin, which was from barley, indoor dust and rabbit dung.

### 2.2.2 *Fungal strains*

The strains that were analyzed in this study are shown in Table 2.1. All strains were maintained on MEA medium [16] and laboratory stocks were made by storing agar plugs, containing mycelium and spores, at 4 °C.

**Table 2.1** Identity, collection number, environment and origin of the fungi analyzed.

| Fungal strain | Code/Collection no. | Environment | Origin |
|---|---|---|---|
| *Aspergillus carbonarius* | ITEM 5010 | Grapes | Italy |
| *A. niger* | IBT 32334 | Indoor dust | Denmark |
| *A. ostianus* | 14 (internal code) | Garden Park Waste | Denmark |
| *Fusarium culmorum* | IBT 41924 | Barley | Denmark |
| *F. graminearum* | IBT 41926 | Barley | Denmark |
| *F. poae* 1 | IBT 41928 | Barley | Denmark |
| *F. poae* 2 | IBT 41929 | Barley | Denmark |

| Fungal strain | Code/Collection no. | Environment | Origin |
|---|---|---|---|
| *F. poae* 3 | IBT 41930 | Barley | Denmark |
| *F. sporotrichioides* | IBT 41927 | Barley | Denmark |
| *Geomyces sp.* | IBT 32267 | Garden Park Waste | Denmark |
| *Gliocladium* sp.-1 | IBT 41939 | Garden Park Waste | Denmark |
| *Gliocladium* sp.-2 | IBT 41940 | Garden Park Waste | Denmark |
| *Penicillium allii* | IBT 32332 | Indoor (garlic/onion) | Denmark |
| *P. coprobium* | IBT 3071 | Barley | Denmark |
| *P. coprophilum* | IBT 3064 | Rabbit dung | Netherlands |
| *P. cyclopium* | 74 (internal code) | Garden Park Waste | Denmark |
| *P. olsonii* | IBT 32326 | Indoor dust | Denmark |
| *P. polonicum* | IBT32328 | Indoor dust | Denmark |
| *P. vulpinum* | IBT 6433 | Rabbit dung | Netherlands |

### *2.2.3 Plate Cultivation and enzyme extraction*

All fungal strains were inoculated on wheat bran (WB) agar medium (30 g/L WB (Finax, Denmark), 15 g/L agar and 1 mL/L trace metal solution (0.1 g/L $ZnSO_4 \cdot 7H_2O$ and 0.05 g/L $CuSO_4 \cdot 5H_2O$)). Incubation was for 7 days at 25 °C, in alternating light and darkness. For each fungal strain 20 agar plugs (4 mm in diameter) with mycelium were cut from the plate. The plugs were placed in a 15 mL falcon tube and 2.5 mL double distilled water (Waters) was added. The vial was shaken for 2 h on a rotational vibrating table (Bie & Berntsen, type 3017) at 150 rpm at room temperature. The liquid extract sample was filtered through a 17 mm, 0.45 µm cellulose acetate filter (National Scientific Company, TN, USA) and stored at -20 °C prior to enzyme screening.

### 2.2.3 *Shake flask cultivation and enzyme extraction*

A liquid preculture medium based on the recipe of MEA was prepared as follows: 20 g/L malt extract (Bacto), 20 g/L lactose, 1 g/L peptone and 1 mL/L trace metal solution (0.1 g/L $ZnSO_4 \cdot 7H_2O$ and 0.05 g/L $CuSO_4 \cdot 5H_2O$). The pH was adjusted to 5.4, and the medium was autoclaved at 121 °C. Inoculation of 20 mL preculture (in 50 mL falcon tubes) was performed by transferring scrapes of spores and mycelium from fungi actively growing on MEA. The precultures were incubated at 25 °C with 200 rpm, for 3 days before use as inoculum for shake flask cultivations.

Liquid medium (LM) for shake flask cultivation was: 3 g/L peptone, 0.5 g/L yeast extract (Difco), 2 g/L $(NH_4)_2SO_4$, 4 g/L $KH_2PO_4$, 0.3 g/L $CaCl_2 \cdot 2H_2O$, 1 mL/L trace metal solution and 0.5 mL/L Tween80, pH adjusted to 5.4. Solid substrates WB (3 g) and finely shredded, dried GPW (3 g) were combined with 150 mL of LM in 500 mL shake flasks for each cultivation. The shake flasks were sterilized by autoclaving at 121 °C, and when they had cooled to room temperature the preculture inoculum was added and incubation initiated at 25 °C with 200 rpm shaking for 5 days. The supernatant was collected by transferring

fermentation broth to 50 mL falcon tubes, which was then centrifuged at 10,000 x G for 25 min. The resulting supernatant was used for enzyme screening.

### 2.2.4 *Enzyme screening*

Extracts from agar plugs were analyzed for enzyme activities on 5 different Azurine cross-linked (AZCL) substrates: Arabinan, arabinoxylan, cellulose, galactan and galactomannan (Megazyme, Bray, Ireland). To prepare the different AZCL assays, a stock solution with phosphoric acid (0.08 M) (Merck, Ortho-Phosphoric acid 85 %), glacial acetic acid (0.08 M) (Merck, 100 %) and boric acid (0.08 M) (Merck) was made using double distilled water. Of the stock solution 144 mL was mixed with 200 mL double distilled water, pH was adjusted to 6 and water was added to a final volume of 500 mL. Agarose, 7.5 g (Litex, HSB 200 Protein grade) was added and the solution was heated in a microwave oven until the agarose had dissolved. For each AZCL assay, 500 mg of azurine bound substrate was presoaked in 96 % ethanol 10 min before use. When the agarose solution had cooled to approximately 65 °C, the AZCL substrate was added while stirring. The suspension was poured into Petri dishes (9 cm diameter) and when solidified, 8 wells (4 mm diameter) were cut in the agar and 30 µL enzyme extract was added to each well. The plates were incubated for 24 h at 30 °C. Activities were determined as the radius of the zone of released azurine dye (the blue halo) around each application well, and area of activity was calculated.

The extracts were also screened for β-glucosidase activity using 5 mM para-nitrophenyl-β-D-glucopyranoside (pNPG) (Sigma Aldrich) in 50 mM sodium citrate (pH 4.8) as the substrate. The assay was carried out in a microtiter-plate format according to [17]. A 10 µL volume of sample supernatant was added to 100 µL substrate in 1.5 mL Eppendorf tubes, and incubated in a Thermomixer® comfort (Eppendorf) at 50 °C for 15 min. At the end of the reaction 60 µL of the reaction volume was transferred to a microtiter plate already containing 100 µL 1 M $Na_2CO_3$ for termination of the reaction. Absorbance at 400 nm was measured in a plate reader (BioTek, EL800). Para-nitrophenol (pNP) was used for preparation of a standard curve. One unit (U) of enzyme activity was defined as the volume of enzyme needed to hydrolyze 1 µmol of pNPG in 1 min.

### 2.2.5 *Synergy in wheat straw hydrolysis*

Crude enzymes extracted from *A. carbonarius* grown in solid cultures (extract from agar plugs) and liquid medium (shake flask cultivation) were analyzed for synergy with crude enzyme extracts of the fungi cultivated in the same manner. Steam exploded and dilute acid pretreated wheat straw (kindly donated by

Biogasol A/S, Denmark) was applied at 5 % solids in the reactions, the cellulose content of the wheat straw was 36 % and the amount of total solids 22 % as seen in table 2.2.

**Table 2.2** Composition of Biogasol steam exploded and dilute acid pretreated wheat straw

| Structural component | Pre-treated wheat straw % DM |
|---|---|
| **Cellulose** | **36** |
| **Hemicellulose** | **18.1** |
| Xylan | 16.6 |
| Arabinan | 1.5 |
| **Klason lignin** | **22.1** |
| **Ash** | **1.3** |
| **Residuals** | **22.5** |
| **Total** | **77.5** |

Synergy of crude enzymes from WB-agar was determined by mixing 400 µL crude enzyme extract of *A. carbonarius* with 400 µL of crude extract from potential synergistic fungi. These 800 µL were added to 5 % wheat straw in combination with 500 µL 0.1 M succinic acid buffer, pH 5.0 and incubated at 50 °C, 1400 rpm (Eppendorf Thermomixer comfort), for 2 days. The crude enzymes from shake flask cultivation were analyzed in the same manner applying 400 µL + 400 µL, and also analyzed for synergy when mixing 200 µL of *A. carbonarius* with 200 µL of potential synergistic crude enzymes. After 2 days incubation the samples were boiled at 100 °C for 10 min, mixed with 45 µL of 10 % sulfuric acid and centrifuged at 16,000 x G, for 10 min. The supernatants were analyzed for released sugars using an Agilent 1100 series high performance liquid chromatography (HPLC) system equipped with an Aminex HPX-87H column (Biorad). Control tests were performed by analyzing the individual crude enzyme extracts applied at 800 µL with 500 µL succinic acid buffer.

## 2.4 Results

### *2.4.1 AZCL enzyme activities*

The general lignocellulose degrading activities were qualitatively determined for each of the fungi grown on wheat bran (WB) agar. From Table 2.3 it can be seen that there is an overall stimulating effect on the arabinoxylan degrading activities. All the fungi analyzed in this study, showed high arabinoxylan degrading activities with an average area of 522.3 $mm^2$ activities. The overall lowest activity was the galactomannan (endo-1,4-β-D-mannanase) degrading activity with an average of 173.4 $mm^2$. Interestingly all the fungi had cellulase activity, except for the *F. sporotrichioides* and the *Geomyces* sp. isolate. However, the analyzed

*Fusarium* spp. isolates had in general a low activity of cellulase, with an average activity area of 65.0 $mm^2$, compared to an average of 179.6 $mm^2$ for the rest of the fungi analyzed. It is furthermore striking that out of 18 fungi analyzed, it is only 6 that have a high arabinan degrading activity (281.3 $mm^2$), *i.e.* two indoor Penicillia, two coprophophilic Penicillia and two fungi isolated from GPW. Overall there were 3 measurements (bolded in Table 2.3) that had a large standard deviation from the mean, which decreases validity of these three results. Two of these occurrences were observed for galactomannan, and by excluding these as 0 activity it would indicate that only half of the fungi can produce endo-1,4-β-D-mannanase when grown on WB-agar. Only four of the isolates did not show any arabinanase activity, these were *A. carbonarius*, *A. niger* and *Gliocladium* sp. 1 and 2. The two coprophilic *P. vulpinum* and *P. coprophilum* had the highest galactanase activity areas of 236 $mm^2$ and 294 $mm^2$, relative to all analyzed isolates. These two isolates were among the few that showed activity in all assays, the others were the isolates *A. niger*, *P. coprobium*, *P. cyclopium* and *P. polonicum*.

**Table 2.3** Area ($mm^2$) of AZCL activity haloes, obtained for enzyme extracts from WB agar plugs for the substrates arabinoxylan, galactan, galactomannan, cellulose and arabinan. Error bars denote standard deviation (SD) (n = 3). Bolded diameters, denotes measurements with large outliers.

| Fungal strain | AZCL Activities ($mm^2$ ± SD) | | | | | Origin |
|---|---|---|---|---|---|---|
| | Arabinoxylan | Galactan | Galactomannan | Cellulose | Arabinan | |
| *A. carbonarius* | 471.6 ± 15.7 | 78.5 ± 0.00 | 176.7 ± 0.00 | 172.9 ± 0.0 | - | Grapes |
| *A. niger* | 738.8 ± 22.5 | 101.1 ± 8.52 | 368.9 ± 15.9 | 201.1 ± 0.00 | - | Indoor dust |
| *P. allii* | 517.6 ± 18.9 | 136.5 ± 13.0 | - | 236.3 ± 16.9 | 119.6 ± 9.25 | Indoor(onion) |
| *P. olsonii* | 218.3 ± 12.2 | 132.7 ± 0.00 | - | 75.9 ± 3.60 | 249.8 ± 6.57 | Indoor dust |
| *P. polonicum* | 630.7 ± 21.1 | 129.7 ± 12.5 | 176.8 ± 9.62 | 189.1 ± 16.9 | 293.7 ± 14.4 | Indoor dust |
| *F. poae* (1) | 346.9 ± 26.9 | 50.3 ± 0.00 | - | 50.3 ± 0.00 | 38.5 ± 0.00 | Barley |
| *F. poae* (2) | 440.6 ± 35.5 | 64.1 ± 11.5 | - | 95.0 ± 0.00 | 50.8 ± 10.3 | Barley |
| *F. poae* (3) | 465.7 ± 35.5 | 80.1 ± 23.3 | - | 96.6 ± 25.5 | 63.6 ± 0.00 | Barley |
| *F. culmorum* | 530.9 ± 0.00 | 108.1 ± 22.4 | - | 84.0 ± 7.78 | 78.5 ± 0.00 | Barley |
| *F. sporotrichioides* | 380.1 ± 0.00 | 73.6 ± 7.03 | 132.7 ± 0.00 | - | 42.4 ± 5.55 | Barley |
| *F. graminearum* | 452.4 ± 0.00 | 50.3 ± 0.00 | 113.1 ± 0.00 | **47.4 ± 34.1** | 78.5 ± 0.00 | Barley |
| *P. vulpinum* | 755.3 ± 39.8 | 236.3 ± 16.8 | 150.3 ± 5.09 | 254.6 ± 11.5 | 335.6 ± 15.2 | Rabbit dung |
| *P. coprophilum* | 645.6 ± 21.1 | 294.3 ± 28.1 | 226.9 ± 0.00 | 201.1 ± 0.00 | 264.7 ± 26.6 | Rabbit dung |

| Fungal strain | AZCL Activities ($mm^2$ ± SD) | | | | | Origin |
|---|---|---|---|---|---|---|
| | Arabinoxylan | Galactan | Galactomannan | Cellulose | Arabinan | |
| *P. coprobium* | 645.6 ± 21.1 | 126.2 ± 9.25 | 180.9 ± 14.9 | 132.7 ± 0.00 | 132.7 ± 0.00 | Barley |
| *Gliocladium* sp._1 | 615.8 ± 0.00 | - | 177.2 ± 19.2 | 151.9 ± 30.9 | - | GPW |
| *Gliocladium* sp._2 | 594.4 ± 30.3 | - | 201.2 ± 10.3 | 193.3 ± 20.6 | - | GPW |
| *A. ostianus* | 660.5 ± 0.00 | 125.2 ± 33.3 | **78.6 ± 55.7** | 136.7 ± 17.2 | 146.9 ± 9.99 | GPW |
| *P. cyclopium* | 615.9 ± 17.9 | 155.5 ± 29.9 | **99.0 ± 70.2** | 209.7 ± 12.2 | 289.6 ± 34.7 | GPW |
| *Geomyces* sp. | 196.9 ± 5.83 | - | - | - | 254.5 ± 11.5 | GPW |

### *2.4.2* β-*glucosidase activities*

Interestingly the two *Gliocladium* sp. 1 and 2 as well as the *P. cyclopium* isolated from GPW produced the highest amount of β-glucosidase of 3.9, 3.4 and 3.8 U/mL, respectively, compared to the other species screened as seen in Fig. 2.1. Aside from these three isolates it was only three of the *Fusarium* spp. isolates that showed a higher activity than *A. carbonarius*. These three were *F. poae* isolate 2 and 3 as well as *F. sporotrichoides* with activities of 1.4, 1.1 and 0.7 U/mL, respectively. All other isolates analyzed had the same level of β-glucosidase activity as *A. carbonarius,* which was relatively low at 0.5 U/mL.

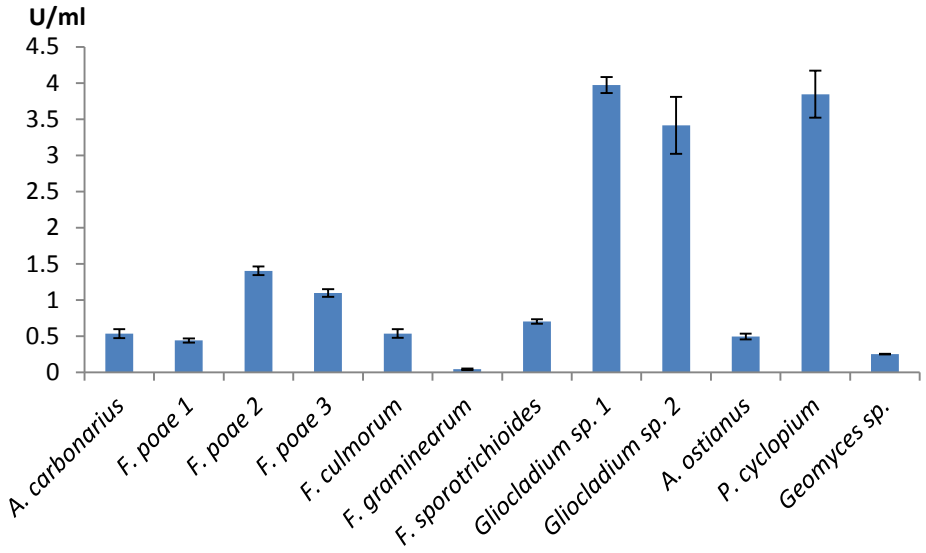


**Figure 2.1** β-glucosidase activities (U/mL) of selected fungi were analyzed based on wheat bran plug extracts. Error bars denote SD (n = 3).

### *2.4.3 Synergy of crude enzyme extracts from solid growth with A. carbonarius*

The application potential of *A. carbonarius* crude enzymes after growth on WB-agar plates was analyzed by hydrolysis of pretreated wheat straw. The apparent glucose release of *A. carbonarius*, when 800 µL crude enzymes were applied, was 0.73 g/L as seen in Fig. 2.2 (A). It is apparent that all the indoor fungi screened have a synergistic effect on the hydrolysis efficiency of *A. carbonarius* with an increase of the glucose release to 2 g/L. By supplementing with the indoor isolate of *A. niger* the highest increase in glucose release of 2.5 g/L was observed. Furthermore two of the coprophilic fungi (*P. vulpinum* and *P. coprophilum*) and one of the GPW fungal isolates (*Gliocladium* sp. 2) were able to elicit as efficient a boosting as that of the indoor fungi. Of all the isolates analyzed for their monoculture efficiency in glucose release (Fig. 2.2, A) it was only *A. niger* that had a higher glucose release (1.64 g/L) than *A. carbonarius*. All other isolates had lower releases of glucose, and only *Gliocladium* sp. 1 and 2 as well as *P. cyclopium* could achieve similar glucose levels as *A. carbonarius*. However, most of the isolates still showed a synergistic effect in combination with *A. carbonarius* as seen in Fig. 2.2 (B). Interestingly the barley isolated *Fusarium* spp. did not induce any significant boosting, except for the *F. sporotrichioides* which could boost the glucose release up to 1.26 g/L.

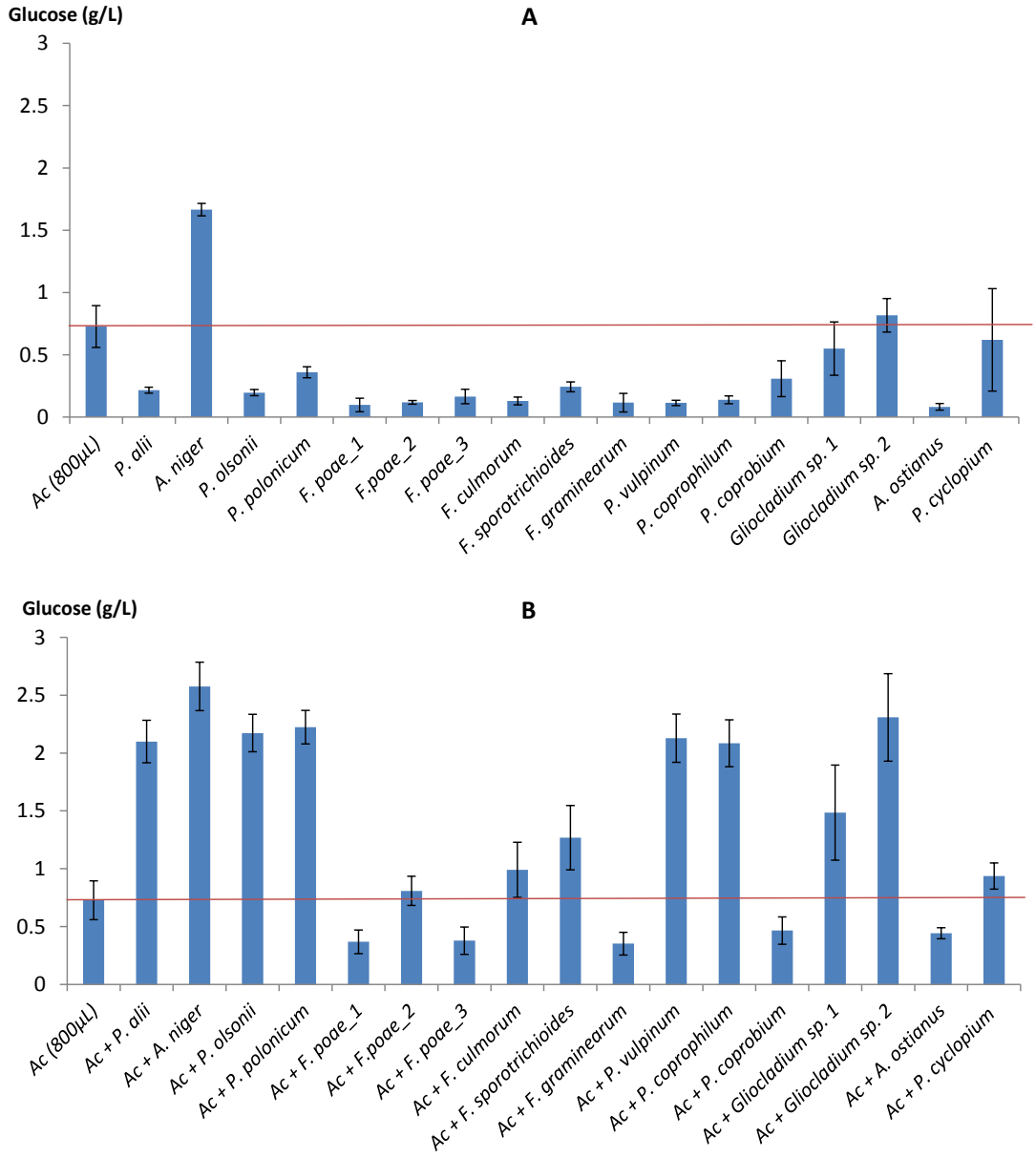


**Figure 2.2** Glucose release after wheat straw hydrolysis. A) For individual fungal crude enzymes at a loading of 800 µL (extract from WB agar plugs). B) Combinations of 400 µL crude enzyme extract of *A. carbonarius* (*Ac*) with 400 µL crude enzymes of the listed fungal isolates. Error bars denote SD (n = 3).

### *2.4.4 Synergy of crude enzyme extracts from liquid growth with A. carbonarius*

The isolates were also analyzed for their ability to boost when the crude enzymes were produced in submerged fermentation. It was clear that the glucose release resulting from application of 800 µL of *A. carbonarius* crude enzymes had increased markedly to 2.90 g/L, see Fig. 2.3 (A). Contrasting the findings for the crude enzymes from solid state cultivations, there were only 4 isolates that showed synergy with *A. carbonarius*. It was apparent that by blending 400 µL of *A. carbonarius* crude enzymes with 400 µL of *F. poae* isolate 1 and *F. graminearum* a significant increase of glucose could be achieved (Fig. 2.3, A)

amounting to 4.64 g/L and 5.49 g/L, respectively. For the two *Gliocladium* sp. isolates there was still an effective boosting of *A. carbonarius*, as seen by the increased glucose release of 4.05 and 3.37 g/L. It was though the *F. graminearum* that proved to be most efficient as a boosting agent when crude enzymes from the submerged cultivation were applied. Combinations with the indoor fungal isolates resulted in a glucose release that was even lower than the *A. carbonarius* control (800 µL) as was apparent for 12 of the combinations. Of the indoor isolates only the addition of *P. polonicum* to *A. carbonarius* resulted in an equal glucose release of 3.05 g/L.

It was further analyzed whether the boosting effects could be observed by lowering the protein loadings to half of what was applied in Fig. 2.3 (A) to a loading of 200 µL + 200 µL crude enzymes. These results show that for 400 µL of *A. carbonarius* the glucose release was 2.07 g/L (Fig. 2.3, B), which point out that there was not a linear correlation between amount of crude enzymes applied and observed glucose release as there was only 2.90 g/L for an 800 µL loading. Furthermore, it appears that the boosting effects observed for the two *Fusarium* spp. at higher protein loadings was not evident when the loadings were halved. For *Gliocladium* sp. 1, there was still a significant boosting effect observed, however, this was the only combination that showed synergy.

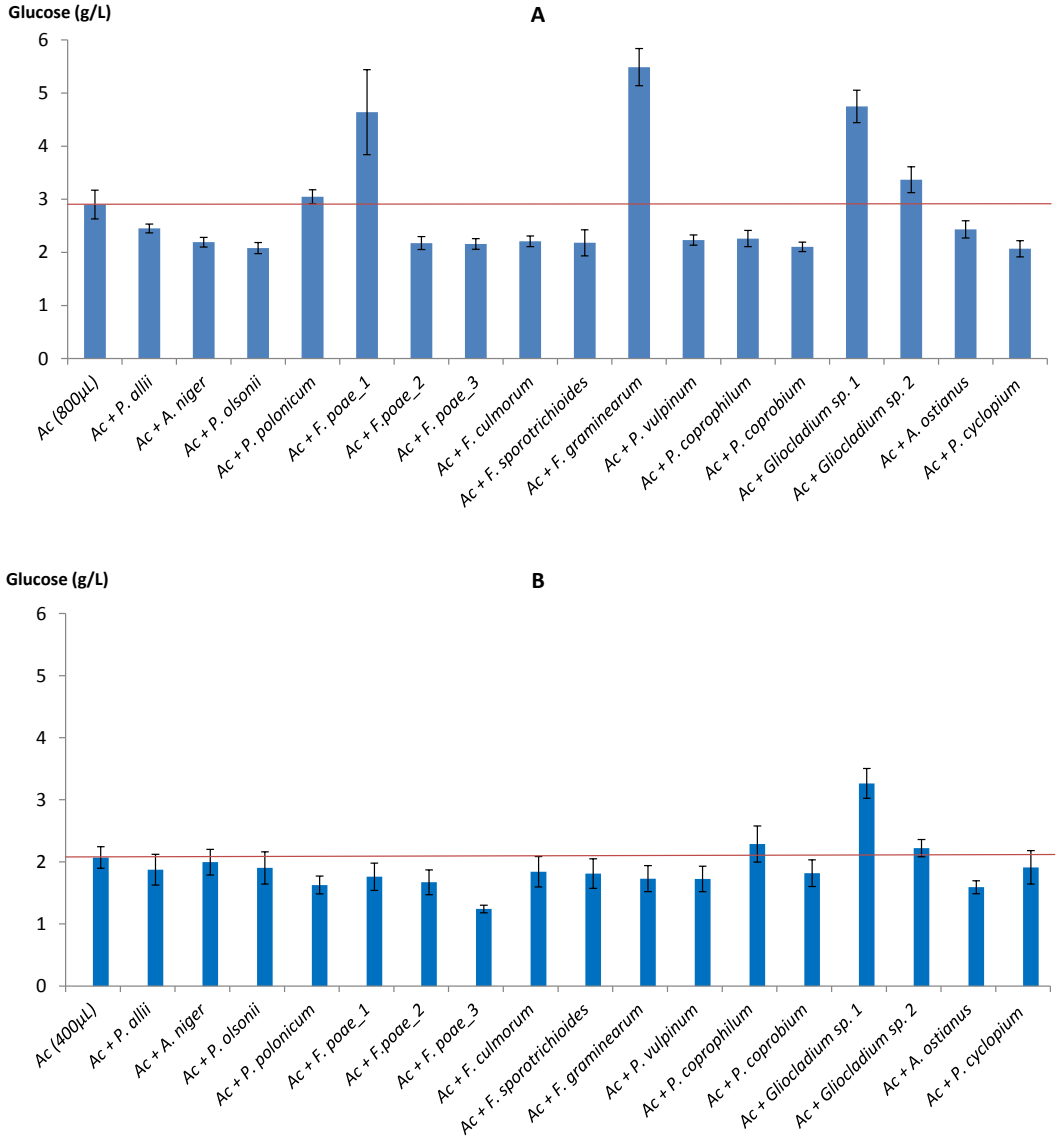


**Figure 2.3** Glucose release after wheat straw hydrolysis by crude enzymes from submerged fermentations. A) Combinations of 400 µL crude enzymes of *A. carbonarius* (*Ac*) with 400 µL of the listed isolates. B) Combinations of 200 µL crude enzymes of *A. carbonarius* (*Ac*) with 200 µL crude enzymes of the listed fungal isolates. Error bars denote SD (n = 3).

## 2.5 Discussion

The overall high endo-xylanase activity observed for all isolates in the AZCL screening could be an effect induced by the high content of glucuronoarabinoxylan (28 %) present in WB [18]. These results provide an insight into which activities can be supplemented to *A. carbonarius* when using crude enzyme extracts from solid WB cultures. The arabinanase activity was lacking in *A. carbonarius* and it was therefore presumed that supplementing with crude enzymes from *P. olsonii*, *P. polonicum*, *P. vulpinum*, *P. coprophilum*, *P. cyclopium*, would induce an increased hemicellulose conversion. All of these isolates except *P. cyclopium*

elicited a synergistic effect in combination with *A. carbonarius* when applying the crude enzymes from solid cultivations. This synergistic effect may have originated from several different types of enzyme activities, among the likely are cellobiohydrolases and oxidative enzymes.

The activity of β-glucosidase observed for *A. carbonarius* 0.5 U/mL is lower than obtained in another study of the same isolate (ITEM 5010) where it was reported to have a high activity of 20 U/mL [5]. However, the low level of activity correlates to a recent study where *A. carbonarius* was grown in liquid minimal medium with 1 % WB as substrate and the crude enzymes were analyzed to have a β-glucosidase activity of 0.1 U/mg protein [19]. Highest activities of β-glucosidase were observed for *P. cyclopium, Gliocladium* sp. 1 and 2 isolates, making these possible boosters of *A. carbonarius* in this setup. A similar activity of 0.84 U/mL for *Gliocladium virens* has been shown, as well as an activity of 246 U/g dry substrate for *Gliocladium* sp. (TUB F-498) [20,21]. Therefore it is not unlikely that the two isolates *Gliocladium* spp. screened in this study could produce high activities of β-glucosidase. Also *P. cyclopium* has been reported to have an activity of 1.1 U/mL in a recent screening study [22].

The crude enzymes obtained from solid WB-agar cultivations were analyzed in wheat straw hydrolysis and most of the isolates were shown to produce a relatively low glucose release around 0.2 g/L. Even with the low glucose release from monocultures observed in the wheat straw hydrolysis (Fig. 2.3, A) it was still possible to screen for synergistic effects of blending crude enzymes, as can be seen in Fig. 2.3 (B). The observed synergies prove that this approach can be used to screen for suitable fungi that can boost *A. carbonarius* or other fungal strains lignocellulose hydrolysis. The *A. niger* and the *Gliocladium* sp. isolates own crude enzymes resulted in higher and equal glucose release as that of *A. carbonarius*, by blending them the glucose release rose to more than what could be ascribed as additive. These isolates and the 5 *Penicillum* spp. could effectively be cultivated for production of a crude enzyme mix to be used as a booster of the crude enzymes from *A. carbonarius*, if a WB containing medium is used. Both *P. vulpinum* and *P. coprophilum* have previously been shown to elicit strong beta-glucosidase, xylanase and pectinase, when assayed on chromogenic substrates [23]. The versatility of these two isolates enzyme apparatus was corroborated by the AZCL screening (Table 2.3) and it may be the reason for their synergistic effect. Whether the identified isolates are capable of boosting the enzyme mixture via co-cultivations remains to be elucidated. The glucose yield from crude enzymes of *A. carbonarius* in solid state can be optimized as it has been shown by Kolasa et al. [5], that a glucose release of 7.5 g/L can be achieved from wheat straw hydrolysis. These results were achieved when growing *A. carbonarius* on 40 g of WB (25.6 %) and sphagnum peat (SP) (15.4 %) medium in solid shake flask cultures, also the whole media was used for extraction of crude enzymes in contrast to this study where only 20 agar plugs were extracted. The scale of

the extraction as well as the amount of nutrients available during cultivation could be the main reasons for this 10 fold difference in activity. Also the lower β-glucosidase obtained here could be affected by the different magnitude of extractions between our study and the one by Kolasa et al. [5]. The WB-agar cultivations can though be used as an efficient screening tool, before scaling to shake flasks and a larger medium.

The crude enzyme production was scaled to shake flasks and evaluated for a liquid medium containing insoluble substrates, from this it could also be evaluated whether the scaling would increase the enzyme titers. For the shake flask cultivations it was apparent that the crude enzyme mixtures were not showing the same trend of synergy as for the crude enzymes from WB-agar with regards to boosting hydrolysis efficiency. This may be a result of the altered cultivation conditions or the altered medium for the cultivation. There was only synergistic effects observed for *F. poae*, *F. graminearum* and *Gliocladium* sp. 1 and 2. Especially for the *Fusarium* spp. the use of a preculture that contained malt extract, could have an inducing effect on their cellulase production in the submerged cultivation. The missing effect of the indoor isolates along with the coprophilic Penicillia goes against what was observed for the WB-agar crude enzymes, thereby indicating that the cultivation and medium drastically affected the crude enzyme composition or the amount of cellulases produced by these isolates.

Also the loading of lower amounts of crude enzymes altered the synergies observed. Furthermore, the non-linear decrease of glucose release for *A. carbonarius* when 400 µL crude enzymes were applied (Fig. 2.3, B) is corroborated in a study by Kumar and Wyman. They applied different cellulase loadings in hydrolysis of pretreated corn stover and even a doubling of the protein loading only resulted in 15 % increase of glucose release [24]. The observed synergy for *Gliocladium* sp. 1 combined with *A. carbonarius* at lower concentration of crude enzymes indicates that there might be a higher concentration of cellulases in the *Gliocladium* sp. 1 supernatant. Protein content in the supernatants was not measured due to the inherent protein content of WB (16 % protein) and GPW (1.9 % protein) which was used as substrate, this extra protein source would have compromised the measurements validity. For future screenings of synergy a high concentration of the crude enzymes should be applied in order to achieve detectable effects.

The difference in enhancing effect of glucose release for the enzymes extracted from two different cultivation processes indicates that the boosting is directly influenced by the medium and whether it is submerged or solid state cultivation. Therefore in future on-site cultivations where two or more cellulase producing strains are to be used, it would be beneficial to determine the optimal combination of medium and growth conditions that yields the most compatible crude enzyme mixtures for increased hydrolysis

efficiency. From this study it appears that *A. carbonarius* could be supplemented by *F. poae*, *F. graminearum* or *Gliocladium* sp. 1, from submerged cultivations in medium containing WB and GPW.

## 2.6 Conclusion

It was observed that an overall strong induction of arabinoxylanase was achieved after cultivation on WB-agar, for all the isolates analyzed in this study. There was also a strong overall expression of galactanase, cellulase and arabinanase activity, however, endo-1,4-β-D-mannanase was only expressed by 10 of the isolates analyzed. *A. carbonarius* had a low crude enzyme activity of β-glucosidase (0.5 U/mL) from the WB-agar extraction, which may explain the boosting observed when supplementing with high β-glucosidase activity crude enzymes. The highest β-glucosidase was observed for *Gliocladium* sp. (3.5 U/mL) and *P. cyclopium* (3.5 U/mL). The same crude enzymes obtained from cultivation on WB-agar were used for studying the complementarity towards the crude enzymes of *A. carbonarius*. For this supplement study it was found that crude enzymes from all of the indoor fungal isolates analyzed could triple the glucose release from wheat straw hydrolysis. The same was observed for two of the coprophilic Penicillia isolates (*P. vulpinum* and *P. coprophilum*), and for *Gliocladium* sp. 2 isolated from garden and park waste. There was also a doubling of the glucose by *Gliocladium* sp. 1, but for the *Fusarium* spp. isolated from barley minor effects were achieved. This method of blending crude enzymes from WB-agar cultivations has applicability as an efficient screening for synergies between crude enzymes. It was further analyzed whether these enhancing effects could be observed in larger scale shake flask cultivations, by applying submerged cultivation using a WB medium supplemented with GPW. From the liquid cultivations the trend of observed synergy between the crude enzymes changed to being only apparent for *F. poae* isolate 1, *F. graminearum* and the two isolates of *Gliocladium* sp. This shows that the cultivation medium and whether it is solid or liquid cultivation has a major impact on the crude enzyme mixtures and their complementarity. For future screening studies of synergies between crude enzymes of other fungi it would therefore be optimal to apply more than one medium in both solid and liquid cultivations. Whether the screening should be performed using solid or liquid cultivation depends on the final application. Furthermore, the synergistic effect was highly dependent on dosage of the crude enzymes from liquid cultivation, evidenced by the loss of synergies when applying 200 µL + 200 µL compared to 400 µL + 400 µL.

## 2.7 Acknowledgements:

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## 2.8 References


[1] Ekman A, Wallberg O, Joelsson E, Börjesson P. Possibilities for sustainable biorefineries based on agricultural residues – A case study of potential straw-based ethanol production in Sweden. Appl Energy 2013;102:299–308.

[2] Sørensen A, Teller PJ, Lübeck PS, Ahring BK. Onsite enzyme production during bioethanol production from biomass: screening for suitable fungal strains. Appl Biochem Biotechnol 2011;164:1058–70.

[3] Rana V, Eckard AD, Teller P, Ahring BK. On-site enzymes produced from *Trichoderma reesei* RUT-C30 and *Aspergillus saccharolyticus* for hydrolysis of wet exploded corn stover and loblolly pine. Bioresour Technol 2014;154:282–9.

[4] Park EY, Naruse K, Kato T. One-pot bioethanol production from cellulose by co-culture of *Acremonium cellulolyticus* and *Saccharomyces cerevisiae*. Biotechnol Biofuels 2012;5:64.

[5] Kolasa M, Ahring BK, Lübeck PS, Lübeck M. Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. Bioresour Technol 2014;169:143–8.

[6] Dwivedi P, Vivekanand V, Pareek N, Sharma A, Singh RP. Co-cultivation of mutant *Penicillium oxalicum* SAU E-3.510 and *Pleurotus ostreatus* for simultaneous biosynthesis of xylanase and laccase under solid-state fermentation. N Biotechnol 2011;28:616–26.

[7] Vinogradova SP, Kushnir SN. Biosynthesis of hydrolytic enzymes during cocultivation of macro- and micromycetes. Appl Biochem Microbiol 2003;39:573–5.

[8] Brijwani K, Vadlani P V. Cellulolytic enzymes production via solid-state fermentation: Effect of pretreatment methods on physicochemical characteristics of substrate. Enzyme Res 2011;2011:860134.

[9] Gupte A, Madamwar D. Solid state fermentation of lignocellulosic waste for cellulase and β-glucosidase production by cocultivation of *Aspergillus ellipticus* and *Aspergillus fumigatus*. Biotechnol Prog 1997;13:166–9.

[10] Gottschalk LMF, Oliveira RA, Bon EPDS. Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. Biochem Eng J 2010;51:72–8.

[11] Visser EM, Falkoski DL, de Almeida MN, Maitan-Alfenas GP, Guimarães VM. Production and application of an enzyme blend from *Chrysoporthe cubensis* and *Penicillium pinophilum* with potential for hydrolysis of sugarcane bagasse. Bioresour Technol 2013;144:587–94.

[12] Sinha M, Sørensen A, Ahamed A, Ahring BK. Production of hydrocarbons by *Aspergillus carbonarius* ITEM 5010. Fungal Biol 2015;119:274–82.

[13] Yang L, Lübeck M, Lübeck PS. Deletion of glucose oxidase changes the pattern of organic acid production in *Aspergillus carbonarius*. AMB Express 2014;4:54.

[14] Weyda I, Lübeck M, Ahring BK, Lübeck PS. Point mutation of the xylose reductase (XR) gene reduces xylitol accumulation and increases citric acid production in *Aspergillus carbonarius*. J Ind Microbiol Biotechnol 2014;41:733–9.

[15] Linde T, Hansen NB, Lübeck M, Lübeck PS. Fermentation in 24-well plates is an efficient screening platform for filamentous fungi. Lett Appl Microbiol 2014;59:224–30.

[16] Samson R, Houbraken J, Thrane U, Frisvad J, Andersen B. Food and Indoor Fungi. 1st ed. CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands; 2010.

[17] Sørensen A, Lübeck PS, Lübeck M, Teller PJ, Ahring BK. β -Glucosidases from a new *Aspergillus* species can substitute commercial β -glucosidases for saccharification of lignocellulosic biomass. Can J Microbiol 2011;650:638–50.

[18] Maes C, Delcour J. Alkaline hydrogen peroxide extraction of wheat bran non-starch polysaccharides. J Cereal Sci 2001;34:29–35.

[19] Meijer M, Houbraken JAMP, Dalhuijsen S, Samson RA, de Vries RP. Growth and hydrolase profiles can be used as characteristics to distinguish *Aspergillus niger* and other black aspergilli. Stud Mycol 2011;69:19–30.

[20] Gomes J, Esterbauer H, Gomes I, Steiner W. Screening of some wild fungal isolates for cellulolytic activities. Lett Appl Microbiol 1989;8:67–70.

[21] Szakacs G, Tengerdy RP. Lignocellulolytic enzyme production on pretreated poplar wood by filamentous fungi. World J Microbiol Biotechnol 1997;13:487–90.

[22] Elshafei AM, Hassan MM, Moursi NM, Elghonemy DH. Screening studies on the formation of β-glucosidase from some filamentous fungi. Adv Food Sci 2009;31:158–63.

[23] Yoon JH, Hong SB, Ko SJ, Kim SH. Detection of extracellular enzyme activity in *Penicillium* using chromogenic media. Mycobiology 2007;35:166–9.

[24] Kumar R, Wyman CE. Effect of enzyme supplementation at moderate cellulase loadings on initial glucose and xylose release from corn stover solids pretreated by leading technologies. Biotechnol Bioeng 2009;102:457–67.

# Chapter 3

## Cellulolytic and xylanolytic activities of common indoor fungi

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# Cellulolytic and xylanolytic activities of common indoor fungi

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## 3.1 Abstract

Mouldy building materials, such as chip wood and gypsum, should be a good source for fungal strains with high production of lignocellulolytic enzymes. Screening of 21 common indoor fungal strains showed, contrary to the expected, that the *Chaetomium* and *Stachybotrys* strains had little or no cellulolytic and xylanolytic activities using AZCL-assays. On the other hand, both *Cladosporium sphaerospermum* and *Penicillium chrysogenum* showed the highest cellulase, β-glucosidase, mannase, β-galactanase and arabinanase activities and would be good candidates for over-producers of enzymes needed to supplement or boost the bioconversion of lignocellulose-rich biomass.

**Keywords**: AZCL enzyme assay, endo-enzymes, wheat bran/sphagnum peat medium

## 3.2 Introduction

Filamentous fungi are among the most efficient degraders of plant biomass, whether it is undesired, as in the deterioration of Army Cotton Canvas, or by design, as in the bioconversion of organic waste material. They are, therefore, the main source of commercial lignocellulase production [1] and high yielding fungal strains are always in demand in the biotech industry [2,3]. The most commonly used organism for commercial enzyme production is *Trichoderma reesei* [1], but it has its limitations. *Trichoderma reesei* produces a high amount of exo-cellulases, but is a poorer producer of e.g. β-glucosidase [4].

Research and screening of filamentous fungi for new high-yielding strains have shown that the original habitat of a strain is important in order to get as specific an enzyme profile as possible. Most of the commercial enzyme producers originate from habitats and substrates rich in lignocellulose, such as compost or agricultural soil [3]. Pedersen et al. [2], showed that *Ulocladium* strains originating from cereal grain (starch) had the highest production of amylase compared to strains from the indoor environment (lignocellulose), which in return had the highest production of arabinanase.

Incidences of fungal deterioration of indoor environments are increasing and can now be seen in most of the western world [5]. It is most obvious on lignocellulose rich materials, such as chip wood or plywood, where the fungal growth can be substantial. It has been shown that there are particular fungal associations between 1) *Cladosporium sphaerospermum* and plywood 2) *Stachybotrys chartarum* and gypsum board and 3) *Ulocladium alternariae* and wallpaper [6]. Mouldy building materials should therefore be a good source for discovery of fungal strains that either produce novel enzyme profiles or over-produce known desirable enzymes.

The purpose with this study was to screen and compare the ten most common fungal species [6] from water-damaged buildings for their production of lignocellulases using *T. reesei* RUT C30 as a reference strain.

## 3.3 Materials and Methods

### 3.3.1 *Fungal strains, media, growth conditions and enzyme extraction*

Twenty-one strains of common indoor fungi and one reference strain were retrieved from the IBT Culture Collection (World Federation for Culture collections WDCM758) housed at Department for Systems Biology, DTU, Denmark. To generate inoculum and check the identity, each strain was inoculated on Campbell's V8

juice agar (V8, [7]) and incubated for 7 days at 25 °C. Fungal identity was confirmed morphologically using Samson et al. [7] and Domsch et al. (2007) [8]. The identity and origin of all fungi are listed in Table 3.1.

**Table 3.1** Fungal strains used in this study with identification numbers and origin.

| Genus | Species | IBT no. | Other no. | Origin |
|---|---|---|---|---|
| ***Aspergillus*** | *versicolor* | 21890 | KD 252-2 | Indoor, USA |
| ***Aspergillus*** | *versicolor* | 28028 | NRRL 3499, IMI 16139 | -, NL |
| ***Chaetomium*** | *elatum* | 41944 | BA Home A | Dust on curtain rail, DK |
| ***Chaetomium*** | *elatum* | 42179 | BA Sample 3009 | Cardboard page from photo album, DK |
| ***Chaetomium*** | *globosum* | 7029 | CBS 148.51, USDA 1042.4 | Stored cotton, USA |
| ***Chaetomium*** | *globosum* | 42177 | Krydsfiner X-a | Plywood, DK |
| ***Cladosporium*** | *sphaerospermum* | 41877 | BAV-KD-C1 | Indoor air sample, DK |
| ***Cladosporium*** | *sphaerospermum* | 41982 | B221/914c | Pipe insulation, DK |
| ***Penicillium*** | *chrysogenum* | 5304 | LH 107 | Indoor air sample, DK |
| ***Penicillium*** | *chrysogenum* | 30128 | DTO 78-E5 | Indoor air sample, DK |
| ***Penicillium*** | *chrysogenum* | 31451 | GR11BA 10b-1-1b | Dust from vacuum cleaner, GL |
| ***Stachybotrys*** | *chartarum* (A) | 9290 | XX | Plaster wall, DK |
| ***Stachybotrys*** | *chartarum* (A) | 14915 | ALK 57 | Gypsum board, DK |
| ***Stachybotrys*** | *chartarum* (M) | 7711 | Dyrup-J | Wood, DK |
| ***Stachybotrys*** | *chartarum* (M) | 40293 | 201 | Indoor, USA |
| ***Stachybotrys*** | *chlorohalonata* | 40285 | 204 | Indoor, USA |
| ***Stachybotrys*** | *chlorohalonata* | 40292 | 103 | Indoor, USA |
| ***Trichoderma*** | *harzianum* | 40876 | TMW 4.1880 | Nutmeg, Grenada |
| ***Trichoderma*** | *harzianum* | 41332 | 16534-a | Indoor air sample, DK |
| ***Trichoderma*** | *reesei* | - | RUT C30 = ATCC 56765 | Cotton duck shelter, Bougainville Island |
| ***Ulocladium*** | *alternariae* | 9058 | ALK 124 | Indoor air sample, DK |
| ***Ulocladium*** | *alternariae* | 41862 | BA 1886 | Wallpaper, DK |

For enzyme production a semi-solid wheat bran/sphagnum peat (WB/SP) medium was made containing (per kg): 256.25 g wheat bran (Finax, Denmark), 153.75 g sphagnum peat (Mosebrug, Denmark) and 590 g water. 40.0 g of WB/SP medium was placed in a 250 mL shake flask and autoclaved. Each fungal strain was inoculated by cutting 3 agar plugs (10 mm in diameter) with spores and mycelium from the V8 plate and transferring these to the shake flask with the WB/SP medium. The shake flasks were incubated at 25 °C and shaken twice a day manually for one week. The experiment was performed in triplicates (66 shake flasks in total).

Each shake flask was added 50 mL double distilled autoclaved water and shaken at 175 rpm overnight at 4 °C. Each extract was filtered through Miracloth into a 50 mL falcon tube and centrifuged at 10,000 g at 5 °C

for 15 min. The supernatant (enzyme extract) was transferred to a clean 50 mL falcon tube and stored at 4°C prior to screening.

### 3.3.2 *AZCL assay preparation and screening*

Six different Azurine cross-linked (AZCL) substrates were used for screening: arabinan, arabinoxylan (wheat and birchwood), HE-cellulose, galactan and β-galactomannan (Megazyme, Bray, Ireland). For each AZCL assay plates were made containing (per 500 mL): 144 mL stock solution, 356 ml double distilled water, 7.5 g agarose (Litex, HSB 200 Protein grade) and 0.5 g AZCL substrate. The stock solution consisted of phosphoric acid (0.08 M) (Merck, Ortho-Phosphorsaure, 85 %), glacial acetic acid (0.08 M) (Merck, 100 %) and boric acid (0.08 M) (Merck) in double distilled water. To prepare the different AZCL assay plates, 200 mL double distilled water was added to the 144 mL stock solution. The pH was adjusted to 6 and double distilled water was added again to give a total volume of 500 mL. Agarose was added and the solution was autoclaved at 120 °C. The AZCL substrate was pre-soaked in 96 % ethanol for 10 min before use. When the agarose solution had cooled to approximately 65 °C, the AZCL substrate suspension was added while stirring. The agarose solution was poured into Petri dishes (90 mm in diam.) and when solidified, 8 wells (5 mm in diam.) were cut in the plates and stored at 4 °C.

For screening 35 µL of enzyme extract was added to each well of the six different AZCL assay plates. The plates were incubated for 24 h at 30 °C. The activity of each enzyme was measured as the radius of the zone of released azurine dye (the blue halo) around each application well. The radius was recorded and converted to area ($mm^2$) and the standard deviation was calculated for each set of triplicates.

### 3.3.3 β-*glucosidase assay and screening*

Screening for β-glucosidase activity was done using para-nitrophenyl-β-D-glucopyranoside (pNPG) 5 mM (Sigma Aldrich) as substrate in 50 mM sodium citrate (pH 4.8). The screening was carried out in a microtiter-plate format according to [9]. A 10 µL volume of enzyme extract was added to 100 µL substrate in 1.5 mL Eppendorf tubes and incubated in a Thermomixer® comfort (Eppendorf) at 50 °C for 15 min. At the end of the reaction 60 µL of the reaction volume was transferred to a microtiter plate already containing 100 µL 1 M $Na_2CO_3$ for termination of the reaction. Absorbance at 400 nm was measured in a plate reader (BioTek, EL800). Para-nitrophenol was used for preparation of a standard curve. One unit (U) of enzyme activity was defined as the volume of enzyme needed to hydrolyze 1 µmol of pNPG in 1 min. Background subtraction was prepared for each sample with 100 µL substrate at reaction temperature. Hereafter, 100 µL stop reagent was added to the Eppendorf tube then 10 µL enzyme for reaction time 15

min. 160 µL of the reaction mixture was then transferred to the microtiter plate and the absorbance was measured at 400 nm.

### 3.4 Results

The AZCL enzyme screenings of the 21 indoor strains were made from crude enzyme extracts from 7 day-old wheat bran/sphagnum peat (WB/SP) medium in solid state fermentations. The standard deviations for three replica were less than 4 % on all analyses, except for *Chaetomium globosum* (IBT 42177) in the arabinanase assay, which was 13 %.

The analyses showed that both *C. elatum* and *C. globosum* had low or no cellulase, mannase or galactanase activities (Fig. 3.1) with the exception of *C. globosum* (IBT 7029) that had an average mannase activity, compared to the highest activities (Fig. 3.1, B). None of the strains of *Stachybotrys chartarum* or *S. chlorohalonata* showed any cellulase, mannase or β-galactanase activity (Fig. 3.1). *Penicillium chrysogenum* showed the highest cellulase and mannase activities (Fig. 3.1, A and B), while *Cladosporium sphaerospermum* showed the highest galactanase activity (Fig. 3.1, C). *Trichoderma*, including the reference strain (*T. reesei* RUT-C30), had in general good cellulase and mannase activities, but very low galactanase activity (Fig. 3.1).

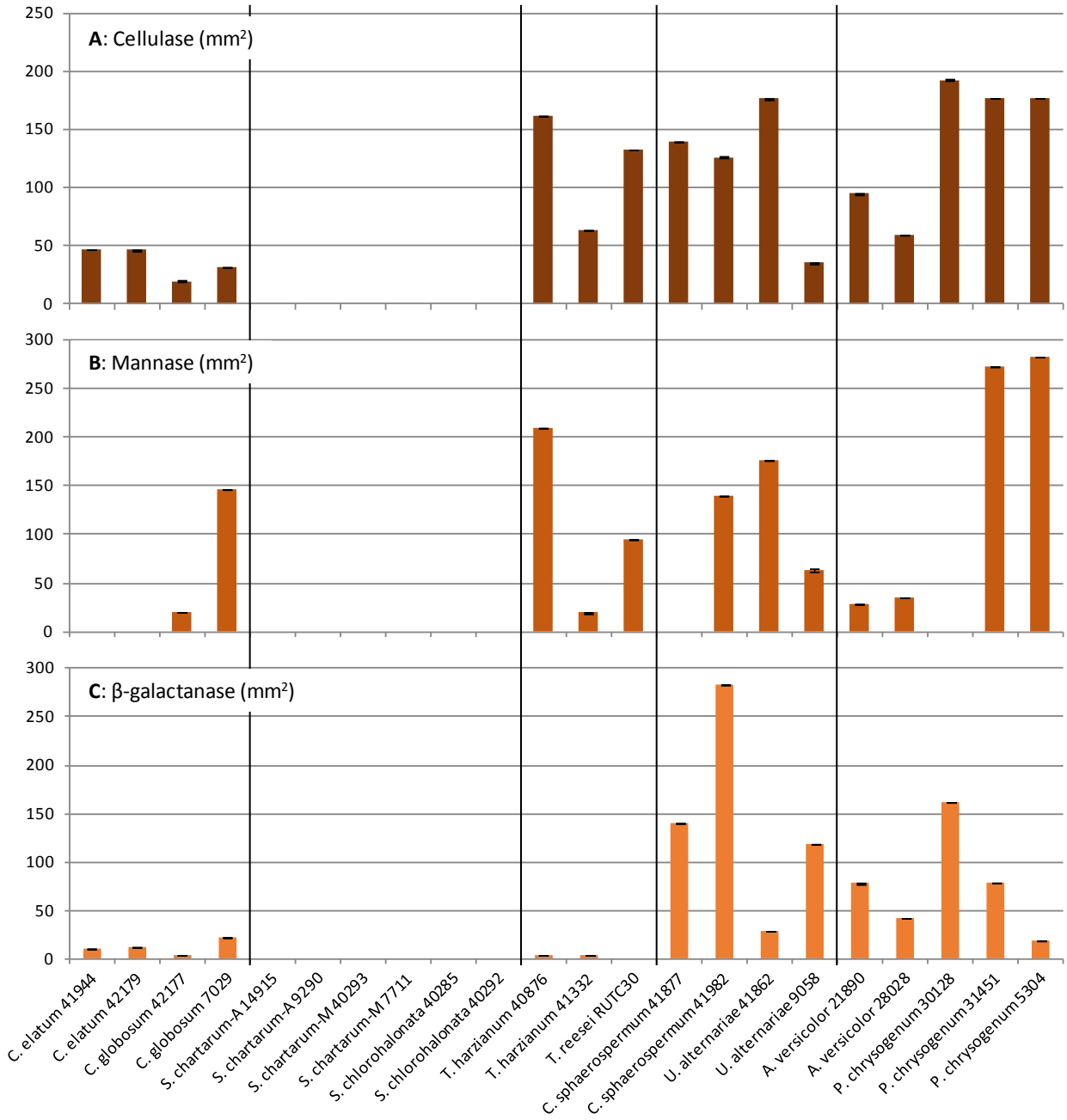


**Figure 3.1** Comparisons of enzyme activity for all 22 fungal strains for A: AZCL-HE-cellulose, B: AZCL-β-galactomannan and C: AZCL-galactan. Error bars show standard deviation (SD) (N = 3).

The β-glucosidase screening again showed that all the *Chaetomium* and *Stachybotrys* strains had a very low activity (0.008 - 0.154 U/mL) compared to the reference strain (*T. reesei* RUT C30) that had an activity of 0.513 U/mL (Fig. 3.2, A). Compared to the high activities (7.823 - 7.653 U/mL) shown by *P. chrysogenum* and *C. sphaerospermum*, both *T. harzianum* and *T. reesei* (RUT-C30) showed low activities (Fig. 3.2, B).

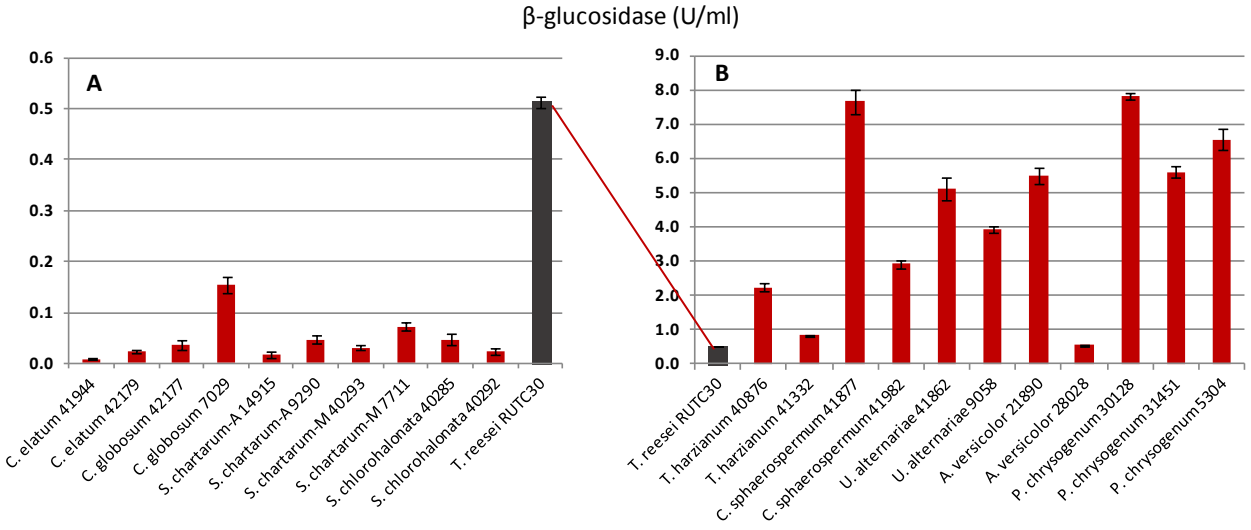


**Figure 3.2** Comparisons of enzyme activity for all 22 fungal strains for β-D-glucopyranoside. A: *Chaetomium* and *Stachybotrys* strains compared with *T. reesei* (gray). B: *T. reesei* (gray) compared with *Trichoderma*, *Ulocladium*, *Cladosporium*, *Aspergillus* and *Penicillium* strains. Error bars show SD (N = 3).

The screening for xylanase production was done on xylan from both wheat and birch and gave similar results (Fig 3.3, A and B). It showed all ten *Chaetomium* and *Stachybotrys* strains as the low producers, while *Aspergillus versicolor* and *T. harzianum* were the high producer for both types of xylanases (Fig. 3.3, A and B). The arabinanase screening, however, showed that neither *T. harzianum* nor *A. versicolor* had any activity, while *P. chrysogenum* had the highest (Fig. 3.3, C). Some of the *Chaetomium* and *Stachybotrys* strains had average activities (e.g. *C. globosum* (IBT 7029) and *S. chlorohalonata* (IBT 40285)), while others showed no arabinanase activity after growth on the WB/SP medium.

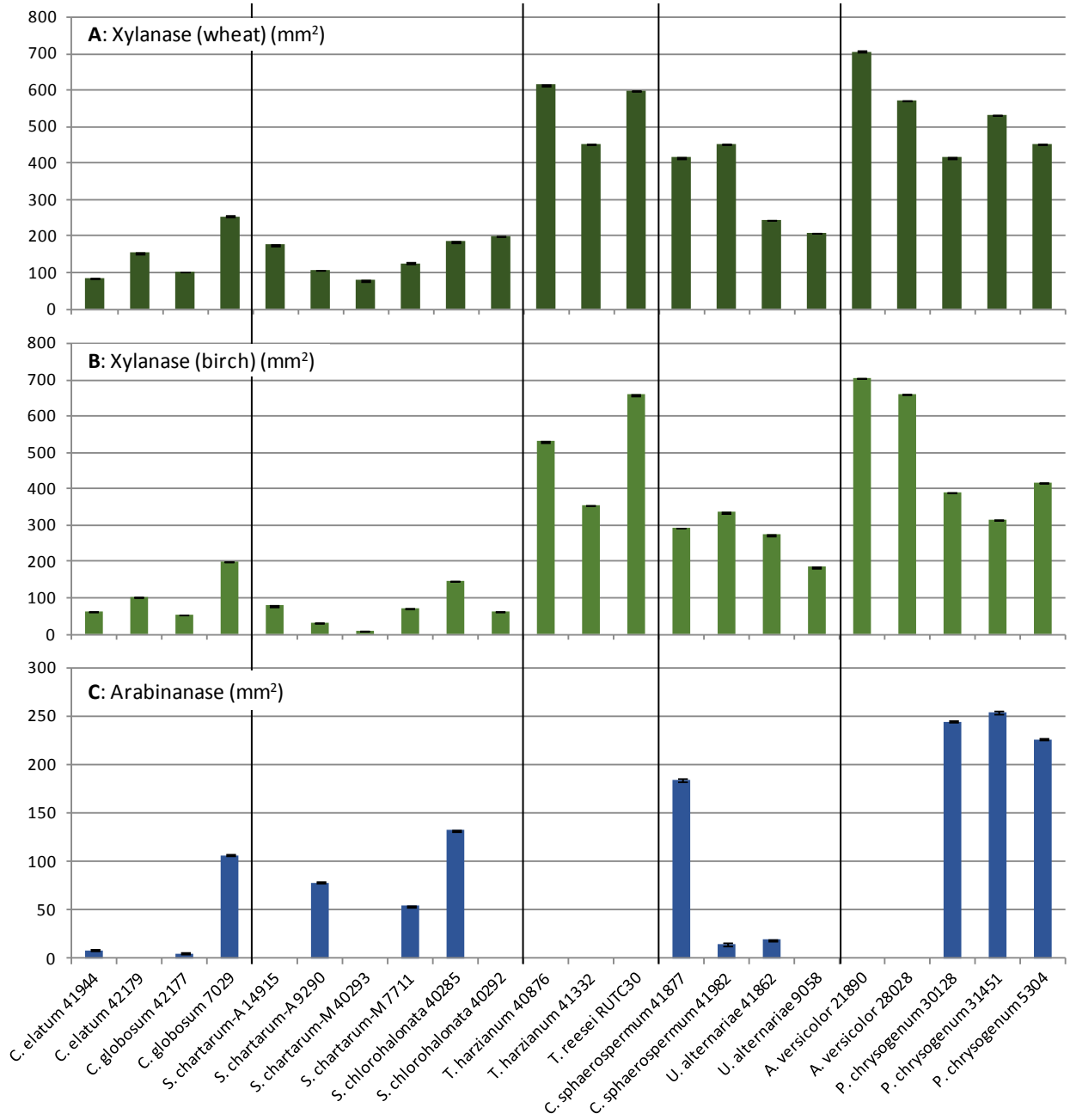


**Figure 3.3** Comparisons of enzyme activity for all 22 fungal strains for A: AZCL-arabinoxylan (wheat), B: AZCL-arabinoxylan (birchwood) and C: AZCL-arabinan. Error bars show SD (N = 3).

A Principal Component Analysis in Fig. 3.4 of all the enzyme screenings shows that *Chaetomium* and *Stachybotrys* (to the left) had similar enzyme profiles, with a general low activity of all tested enzymes. *Aspergillus* and *Trichoderma* (at the bottom) also had similar profiles, both showing high xylanase activities, average cellulase, β-glucosidase and mannase activities and low or no β-galactanase and arabinanase activities. *Penicillium*, *Cladosporium* and to some extent *Ulocladium* (at the top) were similar in having average to high activities of all enzymes tested.

Three strains, *C. globosum* (IBT 7029), *U. alternariae* (IBT 9058) and *T. harzianum* (41332) fell outside their respective groups. *Chaetomium globosum* (IBT 7029) had, overall, a higher activity than the other *Chaetomium* strains, while *U. alternariae* (IBT 9058) and *T. harzianum* (41332) had a lower activity compared to their sibling strains.

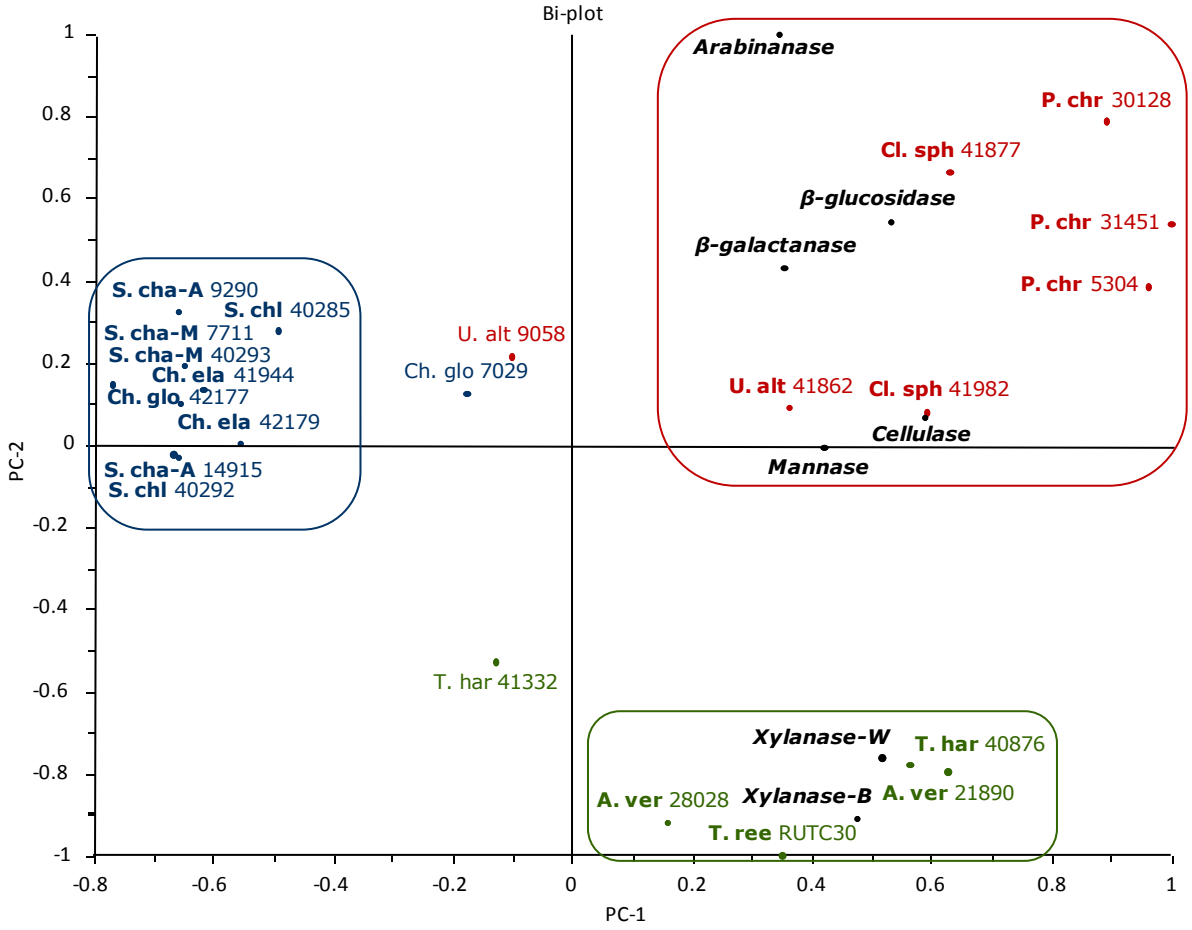


**Figure 3.4** Principal Component Analysis of all 7 enzyme activities and all 22 fungal strains. Arbitrary scales.

### 3.4 Discussion

Wheat bran/sphagnum peat (WB/SP) medium was used since it has been shown to be superior in inducing a broad variety of enzymes [10,11]. WB was also the medium of choice in the study of Pedersen et al. [2], where 50 *Ulocladium* strains were screened for enzyme activity using the AZCL substrates. An attempt to grow the indoor fungi on a similarly composed medium with crushed chipboard/wallpaper instead of wheat bran/sphagnum peat resulted in no growth, probably because the fungi needed a higher water activity to grow on this medium than WB/SP.

The study of Pedersen et al. [2] also showed that there was variation between strains of the same species. This was also seen in this study, for example, with the three *Penicillium chrysogenum* strains that showed similar enzyme profiles in most assays, except for mannose, where *P. chrysogenum* (IBT 30128) had no activity, while the other two strains had the highest activity.

The cellulolytic and xylanolytic activities of *Chaetomium* and *Stachybotrys* have been reported since the 1920s, where the fungi destroyed military equipment and other outdoor cotton fabrics [8,12]. The results in this study show, contrary to the expected, that the *Chaetomium* and *Stachybotrys* strains originating from water-damaged building materials showed little or no cellulolytic and xylanolytic activities using AZCL-assays. Even *C. globosum* (IBT 7029 = CBS 148.51 = USDA 1042.4), which has been used for material testing [13], showed only average activities in the AZCL assays. The fact that these fungi still are able to grow and destroy cellulose-rich building materials, suggests that they have only exo-enzymes and/or membrane bound endo-enzymes, since the AZCL assays screen for extracellular endo-enzymes [14]. Also the low β-glucosidase activity suggests that these enzymes are membrane bound or intracellular. Similar results were seen for *Trichoderma*. The low β-glucosidase activity for *T. reesei* in this study is in accordance with other studies [4] and it has been suggested that this enzyme is membrane bound or intracellular [15].

The other indoor fungi have larger varieties and higher activities of endo-enzymes compared to *Chaetomium* and *Stachybotrys*, which might explain their higher frequency on and lesser specificity for water-damaged building materials [6]. *P. chrysogenum* showed the highest activities for most of the screened enzymes, which might explain its occurrence on most damp indoor surfaces. *C. sphaerospermum* showed a similar result to that of *P. chrysogenum*, however, this fungus is more specialized and also associated with plaster and grouts in bathrooms, due to its ability also to tolerate high fluctuations in humidity [16].

### 3.5 Conclusion

The hypothesis of this study, that mouldy buildings constitute a good source for high cellulase and xylanase producers, was partly proven. Both *Cladosporium sphaerospermum* and *Penicillium chrysogenum* showed high overall enzyme activities and would be good candidates for over-producers of enzymes needed to supplement or boost e.g. *Trichoderma reesei* in conversion of lignocellulosic biomass into bio-fuel. *Chaetomium* and *Stachybotrys* species, on the other hand, showed low enzyme activities suggesting that other carbon sources than cellulose and xylanase are present in e.g. chip wood and gypsum board, which these fungi utilize first.

## 3.6 Acknowledgements

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## 3.7 References


[1] Glass NL, Schmoll M, Cate JHD, Coradetti S. Plant cell wall deconstruction by ascomycete fungi. Annu Rev Microbiol 2013;67:477–98.

[2] Pedersen M, Hollensted M, Lange L, Andersen B. Screening for cellulose and hemicellulose degrading enzymes from the fungal genus *Ulocladium*. Int Biodeterior Biodegradation 2009;63:484–9.

[3] Hansen GH, Lübeck M, Frisvad JC, Lübeck PS, Andersen B. Production of cellulolytic enzymes from ascomycetes: Comparison of solid state and submerged fermentation. Process Biochem 2015;50:1327–41.

[4] Okeke BC. Cellulolytic and xylanolytic potential of high β-glucosidase producing *Trichoderma* from decaying biomass. Appl Biochem Biotechnol 2014;174:1581–98.

[5] Heseltine E, Rosen J. WHO guidelines for indoor air quality: dampness and mould. Druckpartner Moser; 2009.

[6] Andersen B, Frisvad JC, Søndergaard I, Rasmussen IS, Larsen LS. Associations between fungal species and water-damaged building materials. Appl Environ Microbiol 2011;77:4180–8.

[7] Samson R, Houbraken J, Thrane U, Frisvad J, Andersen B. Food and Indoor Fungi. 1st ed. CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands; 2010.

[8] Domsch K, Gams W, Anderson T-H. Compendium of Soil Fungi. 2nd ed. Eching Bei München: IHW-Verlag; 2007.

[9] Sørensen A, Lübeck PS, Lübeck M, Teller PJ, Ahring BK. β -Glucosidases from a new *Aspergillus* species can substitute commercial β -glucosidases for saccharification of lignocellulosic biomass. Can J Microbiol 2011;650:638–50.

[10] Kolasa M, Ahring BK, Lübeck PS, Lübeck M. Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. Bioresour Technol 2014;169:143–8.

[11] Meijer M, Houbraken JAMP, Dalhuijsen S, Samson RA, de Vries RP. Growth and hydrolase profiles can be used as characteristics to distinguish *Aspergillus niger* and other black aspergilli. Stud Mycol 2011;69:19–30.

[12] Greathouse G, Ames L. Fabric deterioration by thirteen described and three new species of *Chaetomium*. Mycologia 1945;37:138–55.

[13] Reese E, Levinson H, Downing M, White W. Quartermaster culture collection. Farlowia 1950;4:45–86.

[14] Vidal-Melgosa S, Pedersen HL, Schückel J, Arnal G, Dumon C, Amby DB, et al. A new versatile microarray-based method for high-throughput screening of carbohydrate-active enzymes. J Biol Chem 2015;290:jbc.M114.630673.

[15] Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B. Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol Biofuels 2009;2:19.

[16] McGinnis MR. Indoor mould development and dispersal. Med Mycol 2007;45:1–9.

# Chapter 4

## Production of cellylolytic enzymes from ascomycetes; comparison of solid state and submerged fermentation

Review published in Process Biotechnology

# Production of cellulolytic enzymes from ascomycetes; comparison of solid state and submerged fermentation

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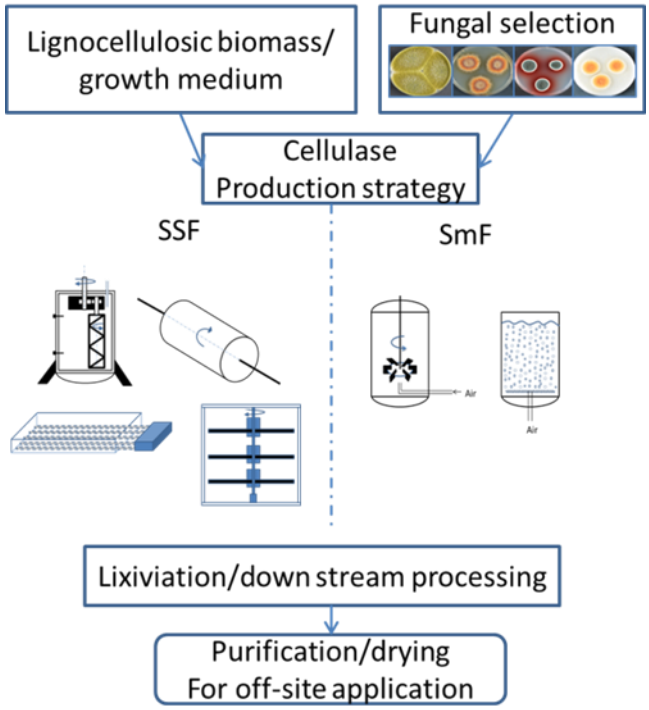
## 4.1 Abstract

Optimizing production of cellulose degrading enzymes is of great interest in order to increase the feasibility of constructing biorefinery facilities for a sustainable supply of energy and chemical products. The ascomycete phylum has a large potential for the production of cellulolytic enzymes. Although numerous enzymatic profiles have already been unraveled, the research has been covering only a limited number of species and genera, thus leaving many ascomycetes to be analyzed. Such analysis requires choosing appropriate media and cultivation methods that ensure enzyme profiles with high specificities and activities. However, the choice of media, cultivation methods and enzyme assays highly affect the enzyme activity profile observed. This review provides an overview of enzymatic profiles for several ascomycetes covering phylogenetically distinct genera and species. The profiles of cellulose degrading enzymes are correlated to the use of submerged culturing and solid state culturing. Even though submerged fermentation (SmF) is the most common method for commercial enzyme production, the use of solid state fermentation (SSF) is praised as a promising way of producing higher enzyme titers compared to SmF. Current comparisons of enzyme activities obtained from SmF and SSF do not account for all variables thereby complicating comparisons and diminishing credibility of conclusions being made. This review aims at providing guidelines for directly comparing cellulolytic enzyme production in SSF with SmF to advance future research of enzyme production.




*Keywords*: Solid state fermentation; Submerged fermentation; Cellulase production; Cellulose conversion; Nomenclature; Ascomycetes

## 4.2 Introduction

One of the promising ways to achieve a sustainable and environmentally friendly energy source is the production of hydrocarbon fuels and bioethanol from lignocellulose [1,2]. There is, however, a lack of large scale industrial biofuel plants in Europe, probably due to challenges with financial viability, economies of scale and lack of political incentives. The economy is especially challenged by the expensive commercial enzymes needed to convert cellulose and hemicellulose in plant biomass into fermentable sugars [3]. In 2004 the market price of commercial cellulase from Alltech was set at $90/kg cellulase [4].

Conversion of plant biomass requires a mixture of different enzymes, including cellulases (e.g. endo-1,4-β-D-glucanase (EG), exo-β-1,4-glucan cellobiohydrolase (CBH) and β-1,4-glucosidase (BG)) as well as the hemicellulases (e.g. exo-1,4-β-xylosidase and endo-1,4-β-xylanase). These enzymes have primarily been derived from ascomycete fungi, in particular *Trichoderma reesei*, *Aspergillus niger* and *Talaromyces pinophilus* (*Penicillium pinophilum*) [5–7]. Commercial enzymes are mostly delivered as mixtures or blends composed of several enzymes, including all or most of the above mentioned enzymes. Such blends can be further optimized by tailoring them for specific biomasses. Tailored blends might also decrease costs pertaining to enzyme production by utilizing an optimal ratio between the enzymes and thereby lowering the enzyme usage [8]. By improving the method of enzyme production a higher yield may be achieved, which would also increase the feasibility of biorefineries. Novozymes, which is the world leading enzyme producer, currently applies submerged fermentation (SmF) for cellulase production. Also the American company Dyadic produces a liquid cellulase product through SmF of *Myceliophthora thermophila* as well as a cellulase powder from SmF of *Trichoderma longibrachiatum*.

Higher yields have been claimed when using solid state fermentation (SSF) [9]. It has also been claimed to be more economically advantageous e.g. for *Clostridium thermocellum* cellulase production in SmF and SSF where the unit costs based on simulated large scale production were calculated to be $40.36/kg cellulase and $15.67/kg cellulase, respectively [4]. The SSF process has been applied for industrial enzyme production of hemicellulases mainly in Japan, using the production organisms *Tr. viride*, *Tr. koningii*, *Tr. reesei* and *A. niger* [10]. Cellulase mixtures are also produced by SSF from the French company Lyven that uses the organisms *Tr. longibrachiatum*, *Rasamsonia emersonii* (previously *Talaromyces emersonii* [11]) and *A. niger*. Other ways to increase yield is to select a new fungal strain from a culture collection or nature with higher activity of the desired enzymes or to genetically engineer a current strain for increased production. Although the engineering of fungal strains is a common approach to increase enzyme production, it is still necessary to know whether the strain performs optimally in SmF or SSF. It is also possible to engineer the enzymes in order to increase their specific activity as well as temperature and pH

stability [12]. Another approach is to reduce the amount of added enzymes as in consolidated bioprocessing (CBP) where the fungal strain simultaneously hydrolyze and ferment lignocellulosic biomass [13].

The purpose of this review is to provide an overview of the process from selection of fungal strains to selection of optimal production methods. The aim is to compare and evaluate the SmF and SSF processes for increased cellulolytic enzyme production, and to put forward recommendations for future enzyme production research. This review does not cover current methods for engineering of enzyme production strains nor will it provide description of alternative processes such as CBP.

### 4.3 Determination of cellulase activity

When it comes to quantification of cellulolytic activity, there are several different assays available. Comparisons between reports on enzyme activity are complicated by the use of different assays and units of activity. There are, however, standardized International Union of Pure and Applied Chemistry (IUPAC) approved methods for determining cellulase activity. These are the filter paper assay (FP), carboxymethyl cellulose (CMC) assay and cellobiase assay [14]. The FP assay has been further standardized by the national renewable energy laboratory [15]. Reproducibility of the CMC and FP assays is challenged by the use of 3,5-dinitrosalicylic acid (DNS) [16], which is used to determine the amount of reducing sugars (glucose) released from the cellulose substrates. This reagent requires boiling for full color development, which may result in partial degradation of reducing sugars. Also the FP assay is highly dependent on the amount of BGs in the enzyme blends being assayed [17]. It has been reported that adding supplemental BGs can increase the reproducibility of the FP assay [18]. With a determined amount of protein, activity is reported as U/g of protein, where U stands for enzymatic units corresponding to amount (µmol) substrate converted pr. minute. If the protein concentration is unknown, the activities will be reported as U/ml of added enzyme solution or as U/g carbon source or substrate (U/gs) in the medium. These three ways of reporting activity are not directly comparable. The activity is sometimes also reported as productivity in U per liter of enzyme solution per hour of production time (U/L/h), giving an idea of the overall potential for the process. In SSF the primary way of reporting activity is by U/gs; this can be used as an assessment of the required substrate to reach such enzyme activities with a given fungus. However, it cannot be used as a measure of productivity, which it is sometimes reported as, since it lacks the measure of time as well as amount of liquid. If the final product of cellulase from SSF is to be applied on-site without lixiviation or drying, as has been suggested [4], the productivity unit U/gs/h is relevant. The amount of added liquid for lixiviation is relevant only if the enzymes are to be freeze dried and used off-site.

The predominant hemicellulolytic enzymes are xylanases, the three best known of these are arabinofuranosidases, endo-xylanases and β-xylosidases. The available assays have variations in both assay conditions (e.g. temperature, duration of incubation or substrate employed) and in the principle of quantification of enzyme activity (e.g. reducing sugars released from substrate, amount of dye released from covalently dyed xylans and measurement of decreases in viscosity or turbidity) [10]. The most commonly used assays apply the measurement of released reducing sugars liberated from insoluble xylans in 1 % solution. The reducing sugars can then be measured by either using DNS reagent [16] or the method of Somogyi-Nelson [19,20]. Furthermore, for increased sensitivity of sugar detection high performance liquid chromatography (HPLC) [21,22] or high performance anion exchange chromatography (HPAEC) can be used [23]. Measurement of monomer sugars via HPLC has been applied for analysis of carbohydrate content of liquid fractions obtained from biomass pretreatment [24]. By using insoluble xylans, these assays become unreliable due to the different degrees of polymerization and substitutions of the xylan polymers. Choice of substrate have been shown to account for variations between 20 laboratories amounting to a standard deviation of 108 % from the mean [25].

Therefore, when attempting to compare enzyme activities across research papers hindrances appear with regards to the assay conditions used. Even when the standardized CMC or FP assays are applied, conditions such as temperature, pH and concentration of buffer as well as the amount of substrate vary between papers [26–30]. In order to obtain comparability between research papers, the conditions just mentioned as well as the unit of enzyme activity must be kept identical.

## 4.4 Enzyme producing ascomycetes

### 4.4.1 *Selection of the best fungal strain*

Commercially interesting ascomycete strains have mostly been isolated from ecological niches rich in agricultural waste (e.g. wheat, rice, maize and sugarcane), since cellulose and hemicellulose are the main components of cereal waste products. Most fungal species are associated with a wide variety of substrata, e.g. tubers, oil seeds or wheat grains in the field [31], however, some of the most promising strains in laboratory screenings have been isolated from hay, straw or husks from cereal plants, compost or agricultural soil (Table 4.1). For example *A. nidulans* is quite common on foods, such as cereal grain, dried fruit, spices and salami [31], but the *A. nidulans* strains used in studies of cellulase enzyme production have been isolated from substrates such as hay and decaying vegetation [32].

One study has shown that while most *Ulocladium* species were able to produce enzymes that degraded amylose, arabinoxylan, β-glucan, cellulose and xylan, both species identity and the source of isolation

(apple, cereals, or indoor environments) impacted the enzyme profile each strain produced [33]. For example, *U. alternariae* isolated from mouldy building materials had the highest arabinase activity (AZCL assays) compared to other species, while strains of *U. curcubitae*, isolated from cereal grains had the highest amylase activity compared to strains isolated from fruit or mouldy building materials. Another study showed that a number of Penicillia from prairie grass were very good producers of cellulases and xylanases [5]. One of the most efficient strains selected from that study was *P. brasilianum* (IBT 20888) [34], but other related species, such as *P. simplicissimum* and *P. pulvillorum,* also showed high cellulase production [35]. The most widely applied cellulase producing ascomycete *Tr. reesei* Rut-C30, was obtained through mutagenesis of the wildtype QM6a, originally named *Tr. viride* and isolated from a cotton canvas army tent on the Solomon Islands [36]. Table 4.1 presents substratum types and climate areas for the fungal species described in this review.

**Table 4.1** Overview of current taxonomical names applied to the fungal strains described in this review, as well as substratum and climate distribution of the fungi. References for each strain can be found in Table 4.2 and 4.3.

| Original fungal name | Correct fungal name[1] | Substratum | Climate |
|---|---|---|---|
| *Acremonium cellulolyticus* | *Talaromyces cellulolyticus* | Soil | Subtropical, humid |
| *Ac. zeae* | *Ac. zeae* | Corn seed | Subtropical, humid |
| *Aspergillus awamori* | *A. niger* | Rice fermentation | Subtropical, humid |
| *A. flavus* | *A. flavus* | Soil (decaying fruit and vegetable) | Tropical, humid |
| *A. foetidus* | *A. niger* | Soil | ? |
| *A. fumigatus* | *A. fumigatus* | Soil (decaying vegetation); leaves; wood chip | Subtropical, semi-arid |
| *A. kawachii* | *A. luchuensis* | Rice fermentation | Subtropical, humid |
| *A. nidulans* | *A. nidulans* | Soil (decaying vegetation); hay; peanut shell | Subtropical, humid |
| *A. niger* | *A. niger* | Soil (agricultural field); black pepper | Subtropical, humid |
| *A. oryzae* | *A. oryzae* | Rice fermentation | Subtropical, humid |
| *A. saccharolyticus* | *A. saccharolyticus* | Soil (lion-ant hole); wooden seat | Temperate/subtropical, humid |
| *A. sydowii* | *A. sydowii* | Compost | Tropical, humid |
| *A. terreus* | *A. terreus* | Soil (rice paddy) | ? |
| *A. tubingensis* | *A. tubingensis* | Soil (agricultural field) | Subtropical, semi-arid/humid |
| *A. ustus* | *A. ustus* | ? | ? |
| *Chaetomium cellulolyticum* | *C. virescens* | Soil (wheat straw) | Subtropical, humid |
| *C. thermophilum* | *C. virescens* | Soil; dung | Subtropical, humid |
| *Fusarium chlamydosporum* | *F. chlamydosporum* | Soil (decaying wood) | Subtropical, humid |
| *F. oxysporum* | *F. oxysporum* | Cumin | ? |
| *Melanocarpus albomyces* | *M. albomyces* | Compost | Subtropical, humid |
| *Mucor circinelloides* | *M. circinelloides* | Soil (decaying wood; corn) | Temperate, humid |
| *Neurospora crassa* | *N. crassa* | ? | ? |
| *N. sitophila* | *N. sitophila* | Wheat straw (steam exploded) | ? |
| *Paecilomyces themophila* | No valid name | Decaying wood in lake | Temperate, semi-arid |
| *Pa. variotii* | *Pa. variotii* | Soil (grassland) | Tropical, humid |

| Original fungal name | Correct fungal name[1] | Substratum | Climate |
|---|---|---|---|
| *Penicillium chrysogenum* | *P. chrysogenum* | Soil (wheat straw); wood chip | Subtropical/tropical, humid |
| *P. citrinum* | *P. citrinum* | Rice straw compost; soil (landfill site) | Subtropical, humid |
| *P. decumbens* | *P. decumbens* | Rice straw compost | Subtropical, humid |
| *P. echinulatum* | *P. echinulatum* | Gut of furniture beetle | Subtropical, humid |
| *P. funiculosum* | *Ta. funiculosus* | Mercury-treated fabric | Subtropical, humid |
| *P. janthinellum* | *P. janthinellum* | Soil (pea field; forest); decaying wood | Temperate/subtropical, humid |
| *P. occitanis*[2] | No valid name | Soil | Temperate, humid |
| *P. oxalicum* | *P. oxalicum* | Soil (mangrove) | Tropical, humid |
| *P. pinophilum* | *Ta. pinophilus* | Agricultural waste at cattle farm; radio set | Subtropical/tropical, humid |
| *Rhizopus stolonifer* | *R. stolonifer* | Cassava waste | Subtropical, humid |
| *Scytalidium thermophilum* | *S. thermophilum* | Soil (compost) | Tropical, semi-arid |
| *Thermoascus auranticus* | *Th. aurantiacus* | Soil (salt marshes) | Subtropical, semi-arid |
| *Trichoderma citrinoviride* | *Tr. citrinoviride* | Soil | Tropical, humid |
| *Tr. harzianum* | *Tr. harzianum* | Decayed sugarcane bagasse | Subtropical, semi-arid |
| *Tr. viride/reesei* | *Tr. reesei* | Cotton canvas fabric | Tropical, humid |
| *Tr. viride* | *Tr. viride* | ? | ? |

[1] Several strains were misidentified or had outdated species names, therefore correct taxonomical names have been provided based on the new "one fungus one name rule" [37].
[2] The correct identity of this "species" (nomen nudum) is unknown.

As can be seen from Table 4.1 most of the fungal strains reported in the literature for their production of cellulolytic enzymes have been isolated from substrata containing a lignocellulosic carbon source, often debris of cereal production or from soil of different agricultural fields. It follows that collection of novel cellulase producers should be done in habitats containing cellulosic substrates. Of the subset of fungi shown in this review, the primary geographical origin is the tropics and subtropics and only a few have been isolated in dry, temperate zones. When a good enzyme producing fungal strain has been found, correct identification and registration is important especially for future comparison. Knowledge of fungal databases, such as Index Fungorum or Mycobank, is also important, since fungal names have changed through time and relevant research papers may be omitted, if older or newer names are not used during a literature search. A correct species name is needed if comparisons to other strains or to related species have to be made. The evaluation of fungal strains for this review showed that several strains were either misidentified or had outdated species names according to the new "one fungus one name rule" [37]. For example, some species formerly identified as *Penicillium*, such as *P. funiculosum*, *P. pinophilum* and *P. purpurogenum* [35] have now been transferred to *Talaromyces* as *Ta. funiculosus*, *Ta. pinophilus* and *Ta. purpurogenus* / *Ta. atroroseus* respectively [31,38,39]. Hence, Table 4.1 gives the correct identification (when information exists) for all species/strains described in the review. Identification using classic morphological criteria often requires a specialist, but DNA sequence data have made species identification

easier. However, the widespread use of rDNA sequencing data (28S, 18S, ITS1 and ITS2) is often not sufficient for an unambiguous identification [40,41] and additional house-keeping genes should be sequenced for a correct identification. Wrongly identified species cause confusion regarding comparisons of fungal enzyme producers.

Industrial cellulase production utilize genetic engineered fungal strains able to produce high levels of either a single enzyme class or a specific set of enzymes which can then be supplemented by additional required enzyme activities. The industrial strains are often engineered using random mutagenesis as in the case of *Tr. reesei* Rut-C30 [36], or by more targeted metabolic engineering. Furthermore, approaches using adaptive and directed evolution have provided novel strains with increased cellulase activities or improved functionality of cellulases for both fungi and bacteria [42,43]. With more and more genomes becoming available for ascomycetes it is advantageous to use bioinformatic tools in combination with metabolic engineering to express interesting enzymes. However, this review will not be describing the many approaches that can be applied for genetically optimizing production of cellulases.

### 4.4.2 *Medium composition for enzyme production*

All the required cellulases for cellulose conversion cannot be produced optimally by just one fungal strain, unless genetically manipulated. Genome-sequencing of numerous ascomycetes has shown that a single fungal strain might have most or all of the required genes in their genomes for production of a full cellulose degrading enzyme system. However, to achieve high expression of all genes, several cultivations using different inducing media are required. For example, the cellulase genes of *Tr. reesei* are induced by cellulose, lactose and β-1,2-di-glucoside sophorose [44] whereas the xylanase genes have been shown to be induced by L-arabinose and D-xylose [45]. Furthermore, Häkkinen et al. showed that different CAZymes were expressed when *Tr. reesei* was grown on different lignocellulose substrates [46]. On steam exploded bagasse there was a strong induction of the *cbh1*, *cbh2*, *egl1* and *egl2* genes compared to a significantly low expression when grown on birch xylan. The majority of CAZyme genes were expressed differently on different substrates and some genes were induced by specific xylans indicating that different xylan side chains can affect the gene expression. A temporal expression was also observed for the CAZyme genes in *Tr. reesei*, indicating that as the degradation proceeds, new linkages and components are exposed that requires other enzyme activities [46]. However, the induction of cellulase and hemicellulase production varies among different ascomycete fungi, e.g. the regulation of transcription of the encoding genes in *Tr. reesei* and in *A. niger* is quite different [47]. In *Tr. reesei* the regulation is coordinated by the Xyr1 regulator (the orthologue of XlnR in *A. niger*) [48] and fine-tuned by an array of activators and suppressors of transcription (Cre1, Hap2/3/5, AceI and AceII) [49]. It seems that in *A. niger* the XlnR regulator plays a more

dominant role and co-regulates the expression of cellulases and hemicellulases [47]. In *A. aculeatus* the genes *cbhI*, *cmc2* and *xynIa* are normally induced by cellobiose and cellulose, but it was recently discovered that these genes are not regulated by XlnR but by a novel transcription factor designated the cellobiose response regulator (ClbR). The *cmc1* and *xynIb* genes controlled by XlnR under cellobiose and cellulose induction were also down regulated in the *clbR* deficient strain, indicating that ClbR also interacts with the XlnR regulator [50]. An ideal enzyme production strategy would be to use a single production strain able to produce all the desired enzymes in a balanced ratio when grown in one medium. However, such a strategy would have to rely on genetically modified strains because no such strains have been identified. Hence it is necessary to use different strains or even different species to produce commercial blends of enzymes. Several ascomycetes, e.g. *A. niger*, *A. oryzae*, *Humicola insolens*, *Ta. funiculosus*, *Tr. longibrachiatum*, *Tr. koningii*, *Tr. reesei* and *Tr. viride* [10,51–54], are employed by the industry to achieve optimal production of the necessary cellulases.

The choice of medium for the production of cellulases can highly affect the yield, as well as the cost of the final product. The most economical and environmentally friendly choice would be waste products as substrates containing both carbohydrate and nitrogen sources that can induce high enzyme production. Agricultural waste products and slipstream waste from biorefineries provide an abundance of possibilities for novel low-cost production media [55,56]. It can be argued whether it is advantageous to use purely one type of waste or a mixture of several waste products, such as municipal waste. Industrial waste streams can provide a pure source of waste products, which are cost effective and renewable. Using pure waste products as a substrate would ensure a more homogenous and reproducible production of enzymes, although they are still complex and can be subject to seasonal variations in their composition. Different agricultural wastes (e.g. sugar cane bagasse, corn stover, elephant grass, fruit pomace and straw or bran from wheat, rice or corn) have been applied for enzyme production [28,57–63]. These agricultural residues can also be used as potential biomass sources for biorefineries, and are therefore ideal for a CBP setup. Pretreatment of the substrate is considered a necessity to lower the recalcitrance of the lignocellulosic biomass. The effect of pretreatment might be limited to specific enzymes and fungal species as was shown for SSF where only the endocellulase activity of *A. oryzae* was significantly affected by pretreatment compared to *Tr. reesei* where both the FP, BG and endocellulase was affected [64]. In a study of *A. niger* cellulase production on different agricultural waste residues in SSF, it was shown that wheat bran (WB), which had only been mechanically pretreated, induced higher activities than the alkali or acidic pretreatments [65]. It has also been shown that sterilized WB induced higher β-glucosidase activity for *A. saccharolyticus* compared to pretreated corn stover [56]. In several cases WB has been reported as the substrate that yields the highest enzyme activities of both xylanases and cellulases for many ascomycetes,

compared to other lignocellulosic wastes [66–69]. For instance, *A. niger* (BC-1) grown on WB in solid state settings had an endoglucanase activity of 48.22 U/gs, which was the highest by a margin of 6.36 U/gs compared to other substrates. In the same study, xylanase and BG activities were similar to those obtained using pea-pod waste and rice straw respectively [70]. Also WB gave rise to the richest gene expression profile of *A. oryzae* compared to rice bran and soybean curd [71]. Supplementation with WB has been shown by Steiner et al. (1994) to increase enzyme production for *Ta. purpurogenus* (*P. purpurogenum*) where endoglucanase activity rose from 29.2 U/ml to 43.1 U/ml. This was observed when the original Sigmacell cellulose C-source was supplemented with 25 % WB. The same study also showed that WB induced the highest level of BG activity.

Defined carbohydrates have also been used in media for cellulase production, including avicel, CMC, lactose, sophorose, cellobiose, solka floc, sorbose and xylose [72][54]. For *Ta. cellulolyticus* C-1 (formerly known as *Ac. cellulolyticus* C-1 (FERM P-18508) [73]) solka floc cellulose has been shown to yield the highest FP activity of 3.4 U/ml under submerged conditions compared to 1.2 U/ml and 0.7 U/ml for lactose and cellobiose respectively [74]. The choice of carbon source (C-source) or substrate (C+N-source) can affect the enzyme productivity, but factors such as the concentration as well as the choice of nitrogen source (N-source) can also highly affect enzyme production. Xylanase activity of *Tr. reesei* increased with an increasing concentration of lactose and L-arabinose while the cellulase activity decreased [75]. Another factor that can influence the enzyme production is the size of the inoculum of the fungus. It can be speculated that residual C-source from the inoculum can have an adverse effect, if a different C-source is applied in the production. The use of different C-sources or substrates in enzyme production research challenge the comparability, due to the diverse enzyme expression observed for growth on different media. Comparison of enzyme production is also challenged by differences in growth parameters, enzyme assays and strain variation within species. Furthermore, the optimal medium observed for a given fungal strain with regards to enzyme production might not be optimal for other fungal strains.

### 4.5 Solid state fermentation (SSF)

The SSF process is defined as fermentation with almost no free water, but with substrate moist enough to support growth of microorganisms [76]. Therefore SSF is the process that best mimics the natural habitat of most filamentous fungi compared to submerged fermentation (SmF). The morphology of fungi growing in solid state is of a mycelial form, where both aerial and substrate penetrating hyphae are produced [77]. This characteristic phenotypical growth pattern is common for all types of SSF compared to SmF where the macro-morphology varies from freely dispersed mycelium to loose mycelial clumps and dense pellets [78]. Currently the industrial demand for cellulases is being met by cultivations of filamentous fungi using SmF

technology [79]. It is however being claimed that the yield is higher for SSF than for SmF when comparing the same strain, therefore attention is put on further developing the application of SSF technology for commercial enzyme production [80]. Application of 5 % (wet basis) of a crude enzyme preparation, from SSF using a *Gliocladium* spp. TUB F-498, to lactic acid bacteria ensiling of wilted or non-wilted alfalfa improved fermentation characteristic and stability of the silage as effectively as supplementation with 0.025 % (wet basis) of commercial Celluclast 1.5 L and Viscozyme 120 L. The crude enzyme preparation however could be applied at one fourth the price of commercial enzymes [81].

Some fungal strains might through time have been adapted and domesticated specifically to either SSF or SmF. For example, the domestication of *A. oryzae* in Japan where SSF was used to produce conidia for sake, miso and soy-sauce production [82]. Another sign of domestication of *A. oryzae* is that homologues of the aflatoxin gene cluster are not expressed under conditions that are otherwise favorable for aflatoxin production by the closely related *A. flavus* [82]. The glucoamylase B enzyme of *A. oryzae* has been shown to be secreted only in SSF on rice, but not in SmF containing pure starch as substrate [83]. Low water activity, temperature and physical barriers to hyphal extension have been postulated to be the reasons for higher glucoamylase secretion in SSF. However, the glucoamylase B (*glaB*) has also been shown to be expressed by *A. oryzae* in SSF on wheat kernels and in SmF on extract of wheat kernels. The transcription disappeared in SmF due to high glucose levels only to reappear after 6 h when levels had dropped, indicating a glucose repression in SmF. Maltose was shown to be inducing the *glaB* transcription and could overrule glucose repression [84]. In SSF, the enzymes may be immobilized in the substrate and can therefore only act on the substrate in close proximity, limiting the glucose repression effect. Hence some strains that have been domesticated to SSF may perform less efficiently in SmF and vice-versa. Also the optimal conditions observed in each system may not be conferrable to the other. Comparisons should therefore be made by keeping as many parameters as possible identical, such as temperature, pH, incubation time, substrate and amount of substrate while taking into consideration the capital, operational and product costs.

The two main types of SSF systems for enzyme production differ by the solid phase either being a natural organic material or an inert support material impregnated or coated with liquid growth medium. Both types of solid are insoluble in water but can absorb water into their matrix, thereby providing the required moisture [85]. Selection of the C-source or substrate (C+N-source) is a major aspect of SSF, and for production of enzymes it is necessary to screen a range of C-sources to find the optimal one corresponding to the chosen ascomycete and desired enzyme(s). Alternatively ascomycetes could be screened for optimal growth and production on a desired type of C-source. Also to be considered are the cost and availability of the C-source or substrate. Water activity ($a_w$) of the medium in SSF is a key parameter for mass transfer of

water and solutes across the microbial cell wall, and is therefore important for controlling the fungal metabolism [76]. Choice of the bioreactor type for SSF is dependent on substrate type, process variables as well as the extent of control required. Several types of bioreactors have been designed for small-scale as well as large scale applications of SSF, including the tray reactors (Fig. 4.1, D), packed bed reactors (Fig. 4.1, C) and drum reactors (Fig. 4.1, A and B).

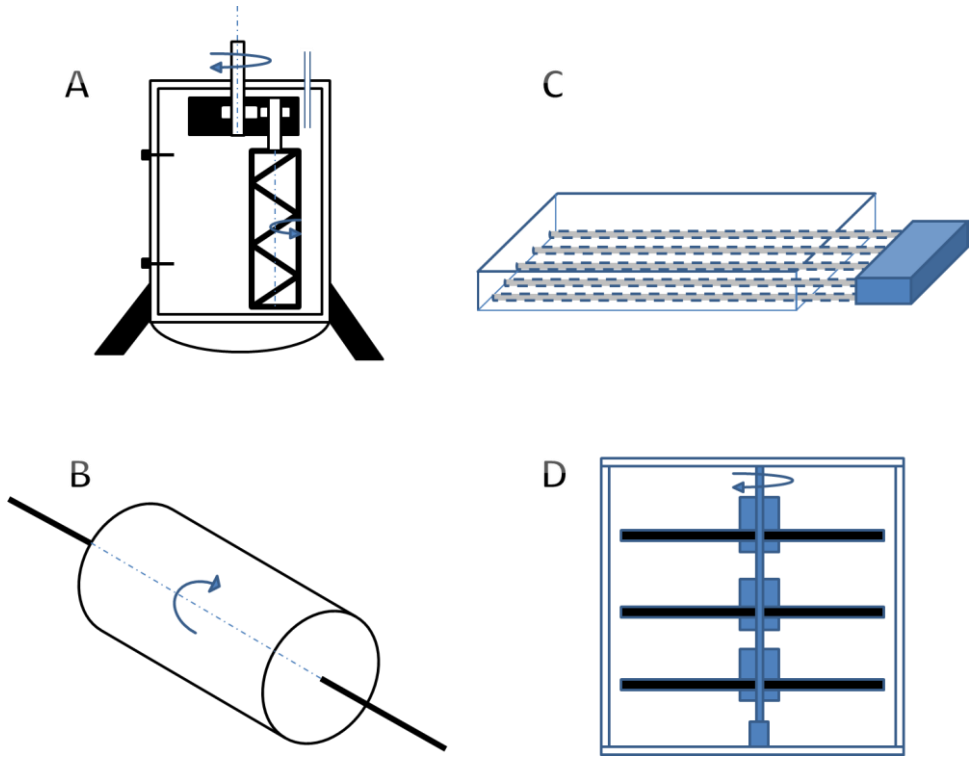



**Figure 4.1** SSF bioreactors, A) standing drum reactor, designed by Lyven, having temperature control, substrate inlet, and stirring [86] B) rotating drum reactor, C) Packed bed reactor with air inlet supplied from the bottom of the tray, D) multiple tray reactor, with rotation.

The advantage of using tray reactors is that the labor intensity is very low if it is run as a continuous system and it can be easily scaled to larger operations, however, the temperature is difficult to control. Recently patents have been filed for novel designs of the tray reactor, in which a single use fermentation tray is fitted into a rigid form, closed with an upper chamber and aeration is supplied between these two portions [87]. For the packed bed reactors (Fig. 4.1, C) the advantages are a larger substrate bed and an easier product recovery, however, heat transfer becomes difficult with larger substrate bed and with axial air gradients there is a risk of drying out the substrate as well as a risk of air channels forming. A novel design of the packed bed reactor has been made in which they suggest aeration through tubes inserted in the reaction mixture from above, and that this air supply should also provide mixing of the solids [88]. Drum

bioreactors can perform with constant mixing thereby ensuring thermal equilibrium, or with intermittent stirring whereby stationary phase is similar to tray fermentors. A disadvantage of the continuously stirred drum bioreactor is that agglomerates may form and shear stress is increased, on the contrary the intermittently stirred has a lowered shear stress, but will not have as effective aeration. Recently a screw bioreactor was designed for selective SSF, using adapted co-cultures of fungi [89]. This design is also intended for a continuous process with slopping back of the biomass. Also the companies, Lyven and Biocon, that produce commercial enzymes through SSF, use agitated solid state systems based on the rotating drum reactor (Fig. 4.1, B). These systems have improved sterility and a means for controlling temperature as well as relative humidity (Fig. 4.1, A) [86,90]. Furthermore, the company Alltech uses a tray configuration for production of SSF crude enzymes containing cellulase, xylanase, phytase and protease to enhance chicken broiler feed [91]. A bioreactor termed "Fermostat" has been developed for SSF with increased control of inlet and outlet of inoculum and substrate, as well as controlled temperature and agitation. The Fermostat was reported to have a production capacity of 81 U/g cellulase and 492 U/g xylanase when cultivating *A. niger* (USM A1) on palm kernel cake and sugarcane bagasse (1:1 ratio), with 75 % moisture at 30 °C for 4 days [92]. Several other designs exist, however, often based on the tray type or drum type. Flasks, packed bed and tray bioreactors are preferentially used for batch SSF, while rotating drum bioreactors are employed for continuous or batch SSF [79].

### 4.5.1 *Advantages and challenges with SSF*

The higher enzyme production in SSF is believed to be the major advantage over SmF and has been associated with a larger biomass and lower product breakdown [93]. Energy expenditure is lower for SSF compared to SmF since there is less water requirements, no mechanical mixing and the downstream processing also requires less energy [79,93]. A significant advantage of SSF would indeed be to use crude wet or dry enzyme preparations without lixiviation. This also facilitates a lower capital operating cost, consequently SSF processes can be considered more economically feasible compared to SmF. However, a combined experimental and theoretical analysis of the economics for polygalacturonase enzyme production by a mutant of *A. carbonarius* show that SmF requires 15-24 % lower capital investment compared to SSF when using alternative downstream processing [94]. The economic analysis was based on the calculations of activities observed after cultivation with two different substrates corn flour and WB for SmF and SSF, respectively. This difference in the media might affect activities observed and the use of WB increases the total production cost compared to corn flour. Another study on cost/benefit analysis of laccase production using *Trametes versicolor* showed that SmF required less capital investment and in addition yielded a higher titer of laccase [109]. In both fermentation set ups, WB was the primary substrate,

however, supplemented by potato broth in the case of SmF [95]. In contrast, a comparative economic analysis of SSF and SmF processes for the production of lipases by *P. restrictum* showed the advantage of SSF compared with SmF [96]. The capital investment for SSF was analyzed to be 56 % of the SmF total cost, and even though activity was reported to be 3 times lower in SSF, product cost was still 41.5 US$/L compared to 130.8 US$/L in the SmF. It has been proposed that the final cellulase product can be used from SSF directly on-site, by mixing solid wet material containing cellulase and organism with a fresh substrate, without concentration or freeze drying [4]. The downstream processing cost would thereby be lowered compared to SmF, however, it may lead to difficulties in the usage of the hydrolysate, since hydrolysis is performed at 50 °C, which is enough to kill mycelium but not fungal spores. Therefore a filtration process would still be required, unless the same strain is to be used in fermentation of the hydrolysate.

The use of SSF ensures a process that resembles the natural environment of most filamentous fungi, and enables the use of solid waste materials or spent low value raw materials [97]. It has been shown that SSF provides a setting with lowered or no catabolite repression, along with a lessened degradation of products by proteases. The exopectinase activity produced by *A. niger* (C28B25) increased in SSF when 40 g/L sucrose was added to the medium, whereas the activity decreased when sucrose was added to the SmF medium [98]. The lack of catabolite repression has though been reported to be relative to the use of inert substrate. It was also found that WB as the inert material provided resistance towards catabolite repression for *A. tamarii* xylanase production even in the presence of 10 % metabolizable sugars, compared to using sugarcane bagasse or corn cobs as the inert material where catabolite repression was observed in presence of 1 % metabolizable sugars [99]. Decreased protease activity has been observed in the SSF process compared to SmF [93], this could be explained by a lower autolysis of fungal hyphae due to immobility in the SSF setting. However, autolysis can also be caused by the temperature gradients in SSF. Water insoluble substrates that would otherwise not be applied in SmF can be used in SSF thereby generating a platform for using cheap and abundant C and N-sources. Furthermore, there is no need for using antifoam chemicals in an SSF setting [100].

The major challenges observed for SSF are issues with scale-up, product purification and fungal biomass determination. Scale-up operations are hampered by the reduced control of online monitoring of process parameters, provision of heat and mass transfer as well as mixing [101]. Mass and heat transfer limitations can occur when the substrate is decomposed thereby giving rise to agglomerated solids and decreased porosity [102]. As a consequence of hyphal growth, the fungi growing in SSF are confronted with gradients of water content and temperature, substrate-air interface and concentration of enzymes and substrate.

Controlling temperature is a major challenge due to the heat produced being proportional to respiration rate [77]. Furthermore, there are difficulties keeping a constant moisture level, so water content needs to be measured and replenished by either saturating the air or adding water directly [97]. Heterogeneity of substrate may also give rise to disadvantages with regards to reproducibility, due to increased risk of batch variations. Control of contamination during long term cultivation can be difficult in SSF, especially for tray fermentor setups without the added enclosure seen in packed bed designs [87] or the drum reactors used by the companies Lyven and Biocon [86,90]. Also due to the heterogeneity and insolubility of the substrate in SSF, it can be difficult to determine if a contamination is present, in contrast to SmF where simple microscopic analysis can determine it.

### 4.6 Submerged fermentation (SmF)

Production of enzymes in SmF has been preferred by the industry due to better parameter control of the whole process. The technology has been developed to a point where the parameters temperature, agitation, aeration, foam and pH among others can be extensively controlled, depending on the specific reactor type [103]. All these factors are very important for the yield of the product of interest. Most filamentous fungi tend to produce spherical pellets when grown in a submerged culture, and this morphological difference compared to SSF provides a possible explanation for the observed enzyme production [78]. The optimal morphology for growth and enzyme production is believed to be when hyphal tips are maximized, and in SmF this is achieved by having smaller pellets or dispersed mycelia [104]. Fungal morphology in submerged cultures is very dependent on the level of shear stress applied. If high agitation rates are used, the morphology will be predominantly damaged hyphae and less agglomerates. At lower agitation pellet formation of varying size will be predominant. Other factors that affect morphology are the mass transfer characteristics, pH value, osmolality and micro solid particles in the media [104,105]. Morphological phenotypes can alter between different species. There is no clear relationship between morphology and productivity of the fungi; some depend on dispersed mycelium whereas others depend on pelleted growth. For *A. niger*, a larger glucoamylase production was found when it grew as large micro-colonies compared to macro-colonies [106]. Therefore the ability to control the morphology of a fungus in submerged culture is important for keeping the product yield high [105].

In SmF the process parameters can be controlled to a higher degree compared to SSF, especially the temperature, which is one of the most important factors, because it affects growth rate, dissolved oxygen tension, rate of medium evaporation, pellet formation and product formation [78]. For shake flask cultivations of *A. niger*, it has been shown that temperature changes can highly affect the amount of EG and BG produced [107]. It can be speculated that for any given ascomycete parameters for optimal enzyme

production are unique, but that closely related fungi would require similar parameters. The most commonly used bioreactors for SmF are stirred tanks (Fig. 4.2, A), which can be used continuously to reduce labor intensity and where oxygen transfer is controllable. Stirred tanks are, however, energy demanding and can be very labor intensive if not run as continuous fermentation. Hydrofoil turbines provide a system for highly viscous mycelial fermentation, however, aeration must remain low to keep the impeller flooded. For more shear sensitive fungal fermentation the bubble and airlift bioreactors (Fig. 4.2, B) are ideal, but they come with a disadvantage of more heterogenous morphology and the airlift is less flexible due to gas injection being linked to performance [108–110].

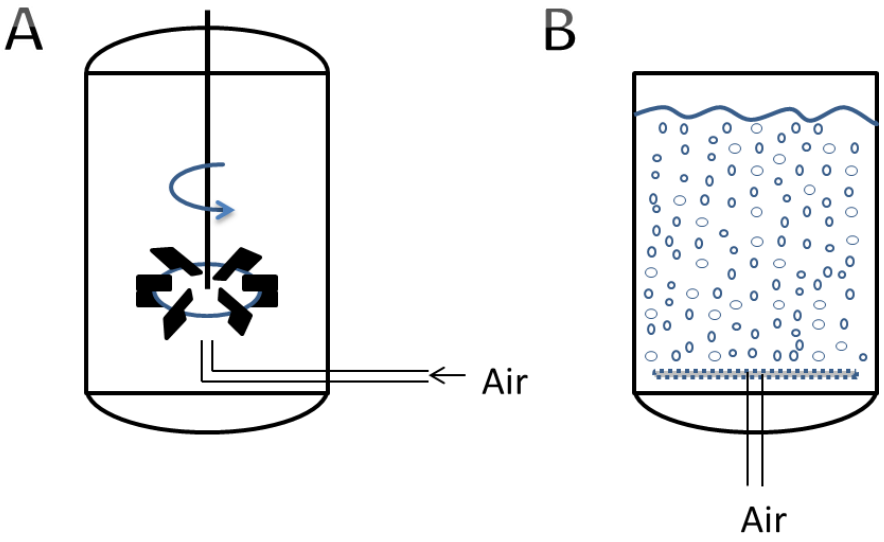



**Figure 4.2** SmF bioreactors, A) Stirred tank with Rushton turbine B) Airlift fermentor with air sparging from the bottom of the tank.

4.5.1 *Advantages and challenges with SmF*

One of the major challenges for production of enzymes in SmF is the multicellular nature of filamentous fungi, which affects the reproducibility of cultivations [111]. Reproducibility of submerged cultivations can be highly affected by the mass transfer of oxygen. If oxygen becomes limited, the filamentous fungi will switch to partially anaerobic metabolism, which might affect the amount of enzyme produced. In submerged cultivations product degradation by proteases can result from autolysis and fragmentation of hyphae, which frequently occur towards the end of the stationary phase [112,113]. Heterologous protein production in industry often utilize protease deficient strains, and recently a study showed that heterologous EG production could be increased by deletion of a major alkaline protease gene [114]. Micro-colonies formed under submerged conditions have been shown to be heterogeneous with regards to

morphology and gene expression level of *A. niger* glucoamylase [115]. Specific morphological forms are required for optimal productivity, therefore the variability of morphology in submerged cultivations can be seen as a challenge towards defining parameters that control and keep the optimal form [78,104]. Achieving optimal productivity also requires an automated setup for supply of nutrients to avoid development of catabolite repression [93].

Major advantages of submerged cultivations are the well-established technological basis for scaling the processes to industrial production capacity. This scalability is mainly due to control of pH, temperature, oxygen and nutrient availability [116]. Another advantage over SSF is that product recovery is simpler for purified/freeze dried cellulase. Reproducibility of submerged cultivations is often not challenged by medium heterogeneity, since it mostly utilizes dissolvable C- and N-sources. However, the use of agricultural residues and waste streams as substrates in the medium is heterogenic in nature and may pose difficulties with respect to reproducibility.

## 4.7 Cellulolytic enzymes produced in SSF compared with SmF

### 4.7.1 *Enzymes produced in SSF*

The enzyme production that has been reported for different species of ascomycetes in SSF and SmF is shown in Table 4.2 and 4.3, respectively. From Table 4.2 it can be seen that the different setups may give significant variations in the enzyme activities observed, for instance the CMCase (EG) activity of the *A. niger* strains vary from 10.77 U/gs to 310 U/gs and for BG activity it varies from 33 to 215 U/gs. This large variation is not observed for FPase or xylanase activities between two of the *A. niger* strains. The variations can be caused by several factors, ranging from variation between strains to the influence of solid content, substrate, incubation time, temperature, enzyme assays and choice of bioreactor. To conclude whether *A. niger* (NS-2) [65] is capable of producing such high amounts of EG compared to *A. niger* (KK2) [61] and *A. niger* (NCIM 548) [9], a standardized procedure for cultivation and enzyme assay needs to be followed. A strain of *A. terreus* (M11) has been shown to produce FPase activity of 243 U/gs, which is the highest activity observed for all strains listed in table 4.2, and 20 fold higher than another *A. terreus* strain having 10.96 U/gs. This large variation could be due to differences in the enzyme assay, since the setup of the assay for the highest activity had an incubation of 30 min at 60 °C and the setup for the lower activity was incubation for 60 min at 50 °C. In both instances 50 mM sodium citrate buffer at pH 4.8-5 was used and reducing sugars were measured with the DNS reagent under reported identical conditions. Another important factor could be the time of growth since the lower value was obtained after 7 days of growth in contrast to 4 days for the high value. Besides *A. terreus* (M11), the strains that produced the highest FPase

activity were *F. chlamydosporum* and *P. chrysogenum* (QML-2). All three were grown only for 4-5 days and produced a surprisingly high activity of 95 U/g and above, compared to *Tr. reesei* (Rut-C30) which was only 12 U/g at the highest reported. This low FP value for *Tr. reesei* (Rut-C30) could be due to the use of old or deteriorated strains.

The xylanase activity varies highly between species, where the largest activity is observed for *Pa. themophila* 18580 U/gs compared to the lowest of 32.7 U/gs observed for *A. awamori*. Another example of differences in the activities measured is seen for the strain of *Th. aurantiacus* (IMI216529) where xylanase activity of 2929 U/gs and 6193 U/gs were measured after growth at similar conditions [117,118]. The assay that was used in both cases applied 1 % oat spelt xylan (Sigma) and measured reducing sugars with DNS. However, Kalogeris et al. (1998) reporting the higher value of xylanase activity prepared their crude enzymes by filtration with an Amicon PM-10, which would concentrate the amount of enzyme thereby yielding higher values [117]. For *P. janthinellum* (NCIM 1171), it is seen that the CMCase activity does not have a large variation even with large changes in solid concentration and carbohydrates in the medium as well as changes in cultivation time. The xylanase activity for this species varies though from 221 U/gs [119] to 800 U/gs [120], this difference should not originate from the assay since both applied the same method [121]. Furthermore, in [120] it is reported that WB and Sigmacell cellulose gives a xylanase activity of 250 U/gs compared to the combination of WB and steam exploded sugarcane bagasse yielding 800 U/gs. This indicates that the addition of steam exploded sugarcane bagasse is beneficial for xylanase production by *P. janthinellum.* There is almost no change in xylanase activity of two *Tr. harzianum* strains analyzed after cultivation of 2 days and 6 days on different substrates, indicating that neither the cultivation time nor the substrate affect the amount of xylanase produced. For *A. fumigatus* (SMN-1) and the two strains of *P. citrinum* shown in Table 4.2, the setup of the SSF uses 10 % solid substrate in the medium, therefore growth of the fungi is realized on a liquid surface compared to the other setups where growth is on a solid moist surface.

**Table 4.2** Enzyme activities measured after solid state fermentation of different ascomycetes under various conditions.

| Strains | Substrate; Solids (%) | T.;Time (°C;days) | Enzyme activity (U/g substrate) Unless otherwise stated (*,**) | | | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | CMC | Avi | FP | BG | Xyl | |
| *Acremonium zeae* (EA0802) | CS; 26 | 25-28; 39 | 0.03* | - | 0.06* | 0.01* | 0.26* | [122] |
| *Aspergillus fumigatus* | WS; 20 | 55; 3 | - | 83 | - | - | - | [123] |
| *A. fumigatus* (SMN1) | WS; 10 | 30; 7 | 1044* | - | - | - | - | [124] |
| *A. nidulans* (MTCC344) | SCB; 40 | 40; 20 | 28.96 | - | - | - | - | [32] |
| *A. nidulans* (SUO4) | SCB; 40 | 40; 20 | 32.59 | - | - | - | - | [32] |
| *A. niger*[¤] (2B.361 U2/1) | GP+OP; 30 | 30; 10 | 5.4 | - | - | - | - | [62] |
| *A. niger*[¤] (2B.361 U2/1) | GP+OP; 30 | 30; 6 | - | - | - | - | 32.7 | [62] |
| *A. niger*[¤] (2B.361 U2/1) | GP; 40 | 30; 1 | 9.6 | - | - | - | 40.4 | [125] |
| *A. niger* (KK2) | RS; 35 | 28; 4-6 | 129 | - | 19.5 | 100 | 5070 | [61] |
| *A. niger* (NCIM 548) | WB+CB+KP; 40 | 30; 6 | 10.77 | - | - | - | - | [9] |
| *A. niger* (NS-2) | WB; 40 | 30; 4 | 310 | - | 17 | 33 | - | [65] |
| *A. niger* | RH; 20 | 37; 3 | - | - | - | - | 5127 | [126] |
| *A. niger* (NRRL 3) | WB+CC; 30 | 35; 4 | - | - | - | 215 | - | [22] |
| *A. saccharolyticus* (CBS 127449) | WB; 30 | 30; 7 | - | - | - | 105* (5.7**) | - | [55] |
| *A. terreus* (M11) | CS; 20 | 45; 4 | 581 | - | 243 | - | 128 | [127] |
| *A. terreus* | RS; 14 | 45; 7 | - | - | 10.96 | - | - | [128] |
| *A. tubingensis* (JP-1) | WS; 20 | 30; 8 | - | - | 0.67 | - | 1478 | [129] |
| *A. ustus* | WB; 20 | 25; 5 | 11.8 | - | 3.8 | 60 | 615.3 | [130] |
| *A. ustus* | RS; 20 | 25; 5 | 12.6 | - | 5.8 | 15.8 | 740 | [130] |
| *Chaetomium virescens*[¤] (NRRL 18756) | SCB; 60 | 40; 4 | 40.5 | - | - | - | - | [27] |
| *Fusarium chlamydosporum* | SCB+WB; 25 | 30; 4 | 281.8 | 182.4 | 95.2 | 135.2 | 4720 | [131] |
| *F. oxysporum* | CS; 20 | 27; 5 | 304 | 4.1 | - | 0.14 | 1840 | [132] |
| *Melanocarpus albomyces* (IIS68) | SCB; 14 | 45; 4 | - | - | - | - | 7760 | [133] |
| *Neurospora sitophila* | SEWS; 25 | 30; 7 | 0.59** | - | 0.17** | 0.42** | - | [134] |
| *Paecilomyces themophila* (J18) | WS; 17 | 50; 8 | - | - | - | - | 18580 | [135] |
| *Pa. variotii* (MG3) | WB+CEb; 30 | 28; 5 | - | - | - | 96.9 | - | [136] |
| *Penicillium chrysogenum* (QML-2) | WB+CSp; 40 | 30; 5 | 370.2 | - | 101.8 | 321.6 | - | [137] |
| *P. citrinum* (MTCC6489) | RB; 10 | 30; 7 | 2.04* | - | 0.64* | - | - | [26] |
| *P. citrinum* (YS40-5) | RB; 10 | 30; 4 | 180.3 | - | 3.8 | 159.1 | - | [28] |
| *P. decumbens* (L-06) | B+WB; 30 | 30; 6 | - | - | 3.9 | - | - | [59] |
| *P. janthinellum* (NCIM 1171) | WB+A; 38.5 | 30; 4 | 688 | - | 11.1 | - | 221 | [119] |
| *P. janthinellum* (NCIM 1171) | WB+SEB; 62.5 | 30; 10 | 600 | - | 16 | 110 | 800 | [120] |
| *P. oxalicum* | WB; 50 | 45; 6 | - | - | - | - | 3.89* | [138] |
| *Rhizopus stolonifer* | CT; 25 | R.T.; 10 | 0.46* | - | 0.26* | 0.41* | - | [139] |
| *S. thermophilum* (MTCC 4520) | RS+WB; 25 | 45; 7 | 26 | 16 | 1.4 | 71 | 182 | [140] |
| *Thermoascus aurantiacus* (IMI 216529) | WS; 20 | 50; 7 | - | - | - | - | 6193 | [117] |
| *Th. aurantiacus* (IMI 216529) | WS; 25 | 49; 8 | 976 | 2.8 | 4.3 | 45 | 2929 | [118] |
| *Trichoderma citrinoviride* | SS; 30 | 30; 10 | 35 | - | 16 | 26 | - | [141] |
| *Tr. harzianum* (F416) | SF; 17 | 33; 2 | - | - | - | - | 438 | [142] |
| *Tr. harzianum* (SNRS3) | RS; 35 | 30; 6 | 111 | - | 6.3 | 173.7 | 433.8 | [30] |
| *Tr. koningii* (AS3.4262) | VW+WB; 50 | 30; 3.5 | 23.8 | - | 6.9 | - | - | [143] |
| *Tr. reesei* (MCG77) | RB; 45 | 25; 8 | - | - | 2.3 | - | - | [144] |
| *Tr. reesei* (QM9414) | RB; 30 | 25; 4 | 94.2 | 99.7 | 11.6 | - | - | [145] |
| *Tr. reesei* (Rut-C30) | KP+WB; 25 | 30; 4 | 21.5 | - | 12.0 | 18.0 | - | [146] |
| *Tr. reesei* (Rut-C30) | WB; 40 | 30; 5 | 103 | - | 5 | 38.1 | - | [80] |
| *Tr. reesei* (SAF3 (MTCC4876) | WB; 40 | 30; 4 | - | - | - | - | 219 | [69] |
| *Tr. reesei* (QM9414/ATCC26921) | RB; 30 | 30; 8 | - | - | 1.2 | - | - | [144] |

| Strains | Substrate; Solids (%) | T.;Time (°C;days) | Enzyme activity (U/g substrate) Unless otherwise stated (*,**) | | | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | CMC | Avi | FP | BG | Xyl | |
| *Tr. viride* (GIM 3.0010) | BP; 35 | 30; 4.75 | 10.31 | - | 5.56 | 3.01 | - | [147] |

¤ Fungal names have been updated to correct taxonomy, original articles use outdated taxonomy.
*Enzyme activity expressed as U/ml of culture supernatant.
**Enzyme activity expressed as U/mg protein in culture supernatant.
*Abbreviations for enzyme activity assays:* CMC (carboxymethyl cellulase, cmcase), Avi (avicel cellulases, avicelase), FP (filter paper cellulase, FPase), BG(β-glucosidase), Xyl (xylanase).
*Abbreviations for substrate*: A (Avicel), B (bagasse), BP (banana peel), CEb (cellobiose), CB (corn bran), CC (corn cob), CS (corn stover), CSp (corn stover powder), CT (cassava tuber), GP (grape pomace), KP (kinnow pulp), OP (orange peel), RB (rice bran), RH (rice husk), RS (rice straw), SEB (steam exploded sugarcane bagasse), SEWS (steam exploded wheat straw), SCB (sugarcane bagasse), SF (sorghum flour), SS (sugarcane straw), VW (vinegar waste), WB (wheat bran) and WS (wheat straw).
*General abbreviations*: ND (not determined), R.T. (room temperature), T. (temperature).
Note: Only cultures with ATCC, NCIM, CBS, QM and NRRL ID's are publically available, so correct identity of the other enzyme producers cannot be verified.

### 4.7.2 *Enzymes produced in SmF*

In Table 4.3 it is apparent that the enzyme activities observed for the same species may vary extensively depending on culture conditions or enzyme assay setup. For example, for *P. janthinellum* the xylanase activity varies from 8.9 U/ml (CRC-87M-115) to 160 U/ml (NCIM 1171). The higher value of 160 U/ml may have been obtained due to a prolonged reaction time of 30 min compared to 5 min for the low values of 8.9 U/ml, in both instances the reaction temperature was 50 °C. Another possible explanation for the variation is the large difference in incubation time, since the low value was obtained after only 1 day cultivation and the higher value after 8 days. It could also be the different xylans used in the enzyme assays, where the higher value is obtained by using oat-spelt xylan and the lower is obtained by using birch wood xylan. It is, however, not possible to determine whether substrate or culture conditions had a major influence on the enzyme activities [119,148,149]. The highest xylanase activity observed is produced by *A. niger* strains grown on wheat straw (6658 U/gs) and rice straw supplemented with WB (620 U/ml). The use of wheat straw as substrate in the medium also produced the highest CMCase activity for *A. fumigatus* (SMN1) of 321 U/ml. However, when the same strain was grown under SSF conditions and with the same substrate, the CMCase activity was 3 times higher resulting in 1044 U/ml [124]. For *F. oxysporum* (F3) wheat straw yielded a higher CMCase activity compared to the same strain grown under similar conditions using WB, however, it is the opposite for BG activity [150]. This indicates that the selection of substrate is important in order to obtain highest enzyme activity, e.g. for *F. oxysporum* in order to maximize the xylanase production wheat straw should be used, and for BG it should be WB. Another striking example of the substrates influence on enzyme production is observed in [151], where *A. niger* (ATCC 14916 originally designated as *A. foetidus*) was shown to produce highest level of xylanase when grown on corn cobs 547 U/ml compared to 159 U/ml from WB growth. The growth conditions were identical as were the enzyme assay conditions.

**Table 4.3** Enzyme activities measured after submerged fermentation of different ascomycetes under various conditions.

| Strains | Substrate (%); Stirring (rpm) | T.; Time (°C;days) | Enzyme activity (U/ml supernatant) Unless otherwise stated (*, **, ***) | | | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | CMC | Avi | FP | BG | Xyl | |
| *Acremonium zeae* (EA0802) | 1 CS; 180 | 30; 9 | 0.09 | - | 0.14 | 0.02 | - | [122] |
| *Aspergillus flavus* (DFR-6) | 2.5 WB; ND | 35; 6 | - | - | - | - | 31.4 | [152] |
| *A. fumigatus* (SMN1) | 1 WS; 130 | 30; 7 | 321 | - | - | - | - | [124] |
| *A. fumigatus* (VTTD-82195) | 6 SF; 750 | 30; 6.6 | - | - | 0.3 | - | 95 | [153] |
| *A. luchuensis*[¤] (FS005) | 2.5 B +0.5 WB; 100 | 37; 3 | 0.55 | - | - | - | 12.5 | [21] |
| *A. nidulans* (ATCC62041) | 1 dB; 200 | 37; 10 | 3.0 | - | 0.26 | 0.65 | - | [154] |
| *A. nidulans* (KK-99) | 2 WB; 200 | 37; 6 | - | - | - | - | 40 | [155] |
| *A. niger*[¤] (2B.361 U2/1) | 2 GP +OP; 200 | 30; 5 | 6.2 | - | - | - | 98 | [62] |
| *A. niger* (A12) | 1 G +1 SCB; 200 | 32; 2 | 0.82 | - | - | - | - | [156] |
| *A. niger* (NCIM 548) | 6 WB +CB +KP; 170 | 30; 6 | 5.54* | - | - | - | - | [9] |
| *A. niger* | 10 WS; 200 | 37; 3 | - | - | - | - | 6658** | [126] |
| *A. niger*[¤] (ATCC 14916) | 3 CC; 150, | 28; 4, | 6.6 | - | - | - | 547.4 | [151] |
| | 3 WB; 150 | 28; 4 | 5.8 | - | - | - | 159.5 | [151] |
| *A. niger* (KK2, KFCC 11285) | 3 RS + 1 WB; 250 | 28; 4 | - | - | - | - | 620 | [157] |
| *A. oryzae* (NRRL 1808) | 3 CC; 150, | 28; 4, | - | - | - | - | 225.6 | [151] |
| | 3 WB;150 | 28; 4 | 1.1 | - | - | - | 85.3 | [151] |
| *A. saccharolyticus* (CBS 127449) | 2 WB; 180 | R.T.; 7 | - | - | - | 6.6 (3.1**) | - | [55] |
| *A. saccharolyticus* (CBS 127449) | 4 WB; 800 | 28; 8 | - | - | - | 339.9 | - | [58] |
| *A. sydowii* | 1 WC; 100 | 40; 6 | 1.3 | - | 1.3 | - | 9.2 | [158] |
| *A. terreus* | 1 OPw + 0.6 CBB; 200 | 30; 12 | 14.1 | - | 0.9 | 10.4 | - | [29] |
| *A. tubingensis* (FSS117) | 1 WS; 150 | 30; 5 | 1.2 | - | - | - | 104 | [159] |
| *Chaetomium virescens*[¤] (CT-2) | 2 CE, 1 starch; 200 | 50; 9 | 2.7 | - | - | - | - | [160] |
| *Fusarium oxysporum* (F3) | 2 WS; 250, | 30; 6, | 26.2 | - | - | 0.6 | - | [150] |
| | 2 WB; 250 | 30; 6 | 12.1 | - | - | 3.4 | - | [150] |
| *Melanocarpus albomyces* (CBS 685.95) | 2 Solka Floc; 250 | 45; 3 | 1160*** | - | - | - | - | [161] |
| *Mucor circinelloides* (NRRL 26519) | 1 Lactose; 200 | 30; 4 | 0.3 | - | - | - | - | [162] |
| *Neurospora crassa* (FGSC 4335) | 5 WS; ND | 30; 4 | 14.2 | 2.8 | 1.33 | 0.3 | - | [163] |
| N. sitophila | 1 SEWS +1 S; 180 | 30; 7 | 0.2** | - | 0.1** | 0.1 ** | - | [134] |
| *Penicillium brasilianum* (IBT 20888) | 2 Solka-Floc; 150 | 30; 9.6 | 98 | 0.18 | 0.68 | 1.09 | - | [5] |
| *P. chrysogenum* (PCL501) | 1 A; ND | 25; 0.5 | 0.7** | - | - | - | - | [164] |
| *P. chrysogenum* (PCL501) | 1 SCP; 100 | 30; 3 | - | - | - | - | 6.1** | [165] |
| *P. chrysogenum* (Q 176) | 1 OSX; 150 | 25; 2 | - | - | - | - | 250** | [166] |
| *P. echinulatum* (2HH) | 1 CE + 1MX + 0.5G; 180 | 28; 8 | - | - | 0.22 | 0.18 | - | [167] |
| *P. janthinellum* (CRC 87M-115) | SCB hydrolysate; 100 | 30; 1 | - | - | - | - | 8.9 | [149] |
| *P. janthinellum* (CRC 87M-115) | CC hydrolysate; 60 | 30; 5.5 | - | - | - | - | 54.8 | [148] |
| | OH hydrolysate; 60 | 30; 6 | - | - | - | - | 55.3 | [148] |
| *P. janthinellum* (NCIM 1171) | 2.5 WB + 1 A; 180 | 30; 8 | 37.2 | - | 1.2 | 6.8 | 160 | [119] |
| *P. janthinellum* | 3 A; 1500 | 30; 4.5, | 1.05 | 0.7 | - | - | - | [168] |
| | | 30; 5.4 | - | - | - | 1.5 | - | [168] |
| *P. occitanis* (Pol6) | 3 egPP; ND | 28; 8 | 21 | - | 23 | - | - | [169] |

| Strains | Substrate (%); Stirring (rpm) | T.; Time (°C;days) | Enzyme activity (U/ml supernatant) Unless otherwise stated (*, **, ***) | | | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | CMC | Avi | FP | BG | Xyl | |
| *Talaromyces cellulolyticus*¤ (CF-2612) | 5 Solka-Floc; 600 | 30; 2.7 | - | - | 346* | 40.3* | - | [170] |
| *Ta. funiculosus*¤ (ATCC 11797) | 0.75 PDC; 200 | ND; 15 | 1.8 | - | 0.35 | 1.8 | - | [174] |
| *Ta. pinophilus*¤ | 3 EGR; 150 | 28; 7 | 2.9 | - | 0.18 | 5.6 | 19.8 | [57] |
| *Ta. pinophilus*¤ (IBT 10872) | 2 Solka-Floc; 150 | 30; 9.6 | 6 | 0.07 | 0.32 | 2.45 | - | [5] |
| *Ta. pinophilus*¤ (NTG III/6) | 3 A + 3 BS; 200-600 | 35; 10 | 11.4 | - | 0.76 | 35.4 | 27.3 | [172] |
| *Trichoderma harzianum* (IOC-4038) | 0.75 PDC; 200 | 30; 4 | 0.55 | - | 0.09 | 0.7 | - | [173] |
| *Tr. harzianum* (E58) | SEA; ND | 28; 6 | 53 | - | 2.3 | - | 1.8 | [174] |
| *Tr. reesei* (QM9414) | 1 A; 300 | 30; 5.75 | 3 | - | - | - | 3.4 | [175] |
| *Tr. reesei* (Rut-C30) | 2 dsWB; 120 | 30; 5 | 64* | - | 5* | 27.1* | - | [80] |
| *Tr. reesei* (Rut-C30/NRRL 11460) | 5 Solka Floc; 350, | 28; 4.4, | 0.63** | - | - | - | 0.53** | [176] |
| | 5 SBP; 350, | 28; 4.4, | 0.6** | - | - | - | 0.39** | [176] |
| | 1.7SBP+3.3aSBP; 350 | 28; 4.4 | 2.9** | - | - | - | 6.3** | [176] |
| *Tr. reesei* (SAF3/MTCC4876) | 1 BX; 200 | 30; 3 | - | - | - | - | 4.75 | [69] |
| *Tr. reesei* (CL-847) | 5 WCE + 2 WB; ND | 25.5; 6.25 | 35 | - | 13.7 | - | 489 | [177] |
| *Tr. reesei*¤ (QM9414) | 1 SCB + 1 WB; 90 | 28; 22 | 0.6 | - | 0.31 | - | 0.62 | [178] |
| *Tr. viride* (NCIM 1051) | 1 SCB; 180 | 30; 10 | 21.8 | - | 0.88 | 0.33 | 69.9 | [179] |

¤ Fungal names have been updated to correct taxonomy, original article use outdated taxonomy.
*Enzyme activity expressed as U/g dry substrate
** Enzyme activity expressed as U/mg protein (in the case of Venegas et al., 2013 the activity was expressed as U/g xylanase)
*** Enzyme activity expressed as: nkat/ml
*Abbreviations for enzyme activity assays:* CMC (carboxymethyl cellulase, cmcase), Avi (avicel cellulases, avicelase), FP (filter paper cellulase, FPase), BG (β-glucosidase), Xyl (xylanase).
*Abbreviations for substrate*: A (Avicel), aSBP (alkaline extracted sugar beet pulp), B (bagasse), BS (barley straw), BX (birch wood xylan), CB (corn bran), CC (corn cob), CE (Cellulose), CS (corn stover), CT (cassava tuber), dB (delignified bagasse), dsWB (destarched wheat bran), EGR (elephant grass), egPP (esparto grass paper pulp), G (glucose), GP (grape pomace), KP (kinnow pulp), MX (methylxanthine caffeine), OH (oat husk), OP (orange peel), OPw (Oil palm waste), OSX (oat spelt xylan), PDC (partially delignified cellulignin), RB (rice bran), RH (rice husk), RS (rice straw), S (sucrose), SBP (sugar beet pulp), SCB (sugarcane bagasse), SCP (sugarcane pulp), SEA (steam exploded aspen), SEB (steam exploded sugarcane bagasse), SEWS (steam exploded wheat straw), SF (sorghum flour), SS (sugarcane straw), VW (vinegar waste), WB (wheat bran), WC (walseth cellulose), WCE (whatman CC41 cellulose), WS (wheat straw) and WSp (wheat straw powder).
*General abbreviations*: ND (not determined), R.T. (room temperature), T. (temperature).
Note that only cultures with ATCC, NCIM, CBS, QM and NRRL identifiers are publically available, so correct identity of the other enzyme producers cannot be verified.

## 4.8 Principles for comparing SSF with SmF

Prévot et al. have made an attempt at directly comparing enzyme production of *Tr. reesei* (Rut-C30) in SmF and SSF by using the same substrate, temperature, incubation time and inoculum size [80]. Challenges arise from parameter control in order to keep the processes as similar as possible, this is evident for Prévot et al. where the amount of substrate in the production medium was 10 g WB for SSF and 2 g WB for SmF. Using WB as a substrate will influence any protein measurement performed on the supernatant or crude extracts as WB itself contain a relatively high amount of wheat protein, thereby complicating comparability of the enzyme assays in which Prévot et al. use an enzyme loading of 5 mg protein pr. assay. They measure a total

protein content of 19.56 mg/g dry substrate in SSF and only 3.17 mg/g substrate in SmF. The enzyme activity observed for 5 mg protein loading show that SmF have higher FP, CMC and BG activities, which is most likely a result of dilution of the SSF cellulases due to the high wheat protein content. Using FP activity (FPU) as a measure for enzyme loading in their assays increases the validity of the comparison, although it is still challenged by using different amounts of substrate in the production medium. CMCase and BGase activity is highest for SSF enzymes when loading with 5 FPU. Hydrolysis potential was analyzed by measuring the glucose and cellobiose released after 72 hours incubation of the enzyme extracts with either Avicel or WB. This showed that SSF enzyme extract could release 7.53 g/L glucose from Avicel compared to 6.78 g/L for SmF when the same FPU was applied. For hydrolysis of WB, the glucose release was 3.39 g/L and 1.41 g/L for SSF extract and SmF, respectively, both applied at 5 FPU. Prévot et al. therefore concluded that the SSF crude extract had a greater efficiency in cellulose saccharification, however, the glucose release data was based on duplicate analysis with no standard deviation given for any of the results. Even the comparison using FPU enzyme loading could be compromised by using a different amount of C-source in the production medium if no enzyme blanks are made. Since the crude enzyme extracts can themselves contain free reducing sugars released from the C-source.

Another comparison of enzyme production by *Ac. zeae* (EA0802) in SSF and SmF showed that highest FPase, xylanase and CMCase activities were found for the enzyme extracts made from SmF compared to SSF [159]. In this study, the SSF reaction was initiated with less mycelium, and substrate was 3 g/L lower than SmF, the added liquid was with $(NH_4)_2SO_4$ as a nitrogen source compared to $NaNO_3$ used for SmF. Also the extract from SSF was made by adding 50 mL of sodium acetate, thereby diluting the amount of enzymes produced [159]. Due to the lack of identical cultivation conditions, further studies should be applied to investigate the optimal process for production of cellulolytic enzymes by *Ac. zeae*. Comparisons of SmF and SSF for the production of cellulase by *A. niger* (NCIM 548), utilized response surface methodology (RSM) to determine optimum conditions [9]. It was reported that cellulase activity in U/gs was 1.95 times higher in SSF than SmF. These activities were obtained from what the RSM predicted to be the optimal production conditions, by varying the factors of cultivation time, pH and C-source concentration. Production of cellulolytic enzymes by *N. sitophila* in SmF and SSF was analyzed by [134] and reported to be better in SSF due to higher activities and more protein production. In this study, the amount of steam exploded wheat straw (SEWS) used in SmF was 1 g compared to 5 g used in SSF, and they furthermore milled the SEWS for the SmF process [134]. In a study of *P. janthinellum* NCIM 1171 enzyme production, different amounts of substrate were applied for SmF and SSF, and the cultivation time was 8 days for SmF and 4 days for SSF [119]. Hence direct comparisons are obscured by the different cultivation conditions. In the study by Saqib et al., CMCase activity was measured to be 3 times higher in the SSF supernatant than the SmF by *A.*

*fumigatus* [124]. The only difference between the two processes was the use of 10 times more substrate in SSF.

In order to compare results of enzyme activity/production, especially SmF directly with SSF, the authors suggest the following considerations are made in future research studies to standardize materials and methods.

- The fungal strain should be properly identified to species and deposited in a recognized culture collection.
- The same fungal strain should be used in all experiments for comparison between SmF and SSF.
- Inoculum size, pH, C- and N-source or substrate concentration, temperature and incubation time must be identical.
- The analysis must be run at optimal solid content and optimal shaking for both SSF and SmF.
- Enzyme activities must be measured using standard assay conditions and must be reported using identical units for both processes, preferentially U/mg protein, if there are no indigenous proteins in the substrate.
- Hydrolysis experiments must be performed, to provide knowledge of application potential, it must be performed with a determined Avicelase, FPU activity or protein loading, if there are no indigenous proteins in the substrate.
- The productivity must be provided in U/L/h or as U/gs/h, depending on the application.

Experiments should be made so that information regarding temperature and pH optimum for the enzyme production is determined as well as optimal cultivation time, in order to determine the enzyme production strategy for any given ascomycete. Technical information regarding particle size, packing density and bed height should be reported in the case of SSF. To fully examine whether SmF or SSF is the best approach, several different set-ups should be analyzed for a given ascomycete in the two processes. The application potential is especially important, since it has been shown that standard activity measurements of commercial enzymes do not correlate well with activity toward plant materials [180]. In this regard a comparison study of the bioreactors for SSF and SmF would provide a lead as to which bioreactors have potential for future development to large scale.

## 4.9 Conclusion

Although many research studies have been made for cellulolytic enzyme production in submerged and solid state fermentation, there is still a need for more direct comparison studies to fully determine the efficiency of the two methods, the advantages and disadvantages during comparisons and whether one or the other

can be recommended as the preferred method. The current comparisons of SmF and SSF have been lacking with regards to defining identical or optimal parameters for each of the processes. Also the comparability across individual research papers is near impossible, due to the variability in growth medium, growth parameters and assay conditions as well as the use of fungal strains that are not available in recognized fungal culture collections. The overview of habitats wherefrom good enzyme producing fungal strains have been isolated, shows that most were isolated from soil or debris connected to agricultural production. This entails that screening for new cellulolytic enzyme producers should be more successful if performed in rural areas containing large amounts of plant material, e.g. agricultural farms or recycling plants.

The application of a standardized setup for parameters would facilitate the determination of whether SmF or SSF is the optimal cultivation condition for specific ascomycete fungal strains by enabling comparison of results through intra- and inter-laboratory trials. Knowledge regarding which process that should be preferred is of great importance with regards to optimizing yield of the enzyme-product and also for determining future research into the technologies of either solid state or submerged fermentation. Regarding the debate surrounding biochemical conversion of lignocellulose, it has been proposed that consolidated bioprocessing (CBP) would be more economical than having a dedicated cellulase production either on site or off-site [181]. This is in line with research concerning biological pretreatment as well as adaptive evolution studies to optimize strains for lignocellulosic conversion [182]. Furthermore the use of optimized microbial consortia could be the advantage needed for CBP to be applied instead of having a dedicated enzyme production. Also several studies apply SSF for cultivation of mixed microbial consortia, indicating a higher potential for use of SSF in a CBP setup [183–187]. However the biological conversion of lignocellulose is still a much slower process than the enzymatic hydrolysis, especially the biological pretreatment of biomass is rate limiting [188]. For CBP, challenges also arise when aerobic microbes (ascomycetes) are used for cellulase production in combination with anaerobic fermentation by *S. cerevisiae* for bioethanol [189]. An onsite enzyme production applying a waste stream from a biorefinery setup as substrate might be just as economical and efficient as a CBP setup, especially with the many SSF bioreactor designs that enable the application of waste streams. Due to the rate limit of a full CBP setup, it is at present therefore still advantageous to produce the cellulases in a separate process using either SmF or SSF combined with physicochemical pretreatment of the lignocellulose substrate. Results of previous research in which the same fungal strains were analyzed indicate that SSF has potential as the process for increased enzyme production using *Tr. reesei* Rut-C30, *A. niger* NCIM 548, *N. sitophila* and *A. fumigatus*. However, it is possible that certain fungal species can have a higher production in SmF compared to SSF, which again emphasizes the need for more direct comparison studies. SSF is a promising technology, which could provide an economical and environmentally friendly production of cellulases, based on the laboratory

scale experiments. However it would require massive restructuring for established companies such as Novozymes A/S to change to SSF for their large scale production. For startup companies, it may be beneficial to utilize the SSF process, considering the amount of novel designs of bioreactors being made available as well as the lowered expenses with regards to product recovery and energy expenditure.

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## 4.11 References


[1] Limayem A, Ricke SC. Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Prog Energy Combust Sci 2012;38:449–67.

[2] Bond JQ, Upadhye AA, Olcay H, Tompsett GA, Jae J, Xing R, et al. Production of renewable jet fuel range alkanes and commodity chemicals from integrated catalytic processing of biomass. Energy Environ Sci 2014;7:1500–23.

[3] Ekman A, Wallberg O, Joelsson E, Börjesson P. Possibilities for sustainable biorefineries based on agricultural residues – A case study of potential straw-based ethanol production in Sweden. Appl Energy 2013;102:299–308.

[4] Zhuang J, Marchant M, Nokes S, Strobel H. Economic analysis of cellulase production methods for bio-ethanol. Appl Eng Agric 2007;23:679–87.

[5] Krogh KBR, Mørkeberg A, Jørgensen H, Frisvad JC, Olsson L. Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes. Appl Biochem Biotechnol 2004;113-116:389–401.

[6] Cianchetta S, Galletti S, Burzi PL, Cerato C. Hydrolytic potential of *Trichoderma* sp. strains evaluated by microplate-based screening followed by switchgrass saccharification. Enzyme Microb Technol 2012;50:304–10.

[7] Singhania RR, Patel AK, Sukumaran RK, Larroche C, Pandey A. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour Technol 2013;127:500–7.

[8] Badhan A, Wang Y, Gruninger R, Patton D, Powlowski J, Tsang A, et al. Formulation of enzyme blends to maximize the hydrolysis of alkaline peroxide pretreated alfalfa hay and barley straw by rumen enzymes and commercial cellulases. BMC Biotechnol 2014;14:31.

[9] Kumar S, Sharma HK, Sarkar BC. Effect of substrate and fermentation conditions on pectinase and cellulase production by *Aspergillus niger* NCIM 548 in submerged (SmF) and solid state fermentation (SSF). Food Sci Biotechnol 2011;20:1289–98.

[10] Haltrich D, Nidetzky B, Kulbe KD, Zupancic S. Production of fungal xylanases. Bioressource Technol 1997;58:137–61.

[11] Houbraken J, Spierenburg H, Frisvad JC. *Rasamsonia*, a new genus comprising thermotolerant and thermophilic *Talaromyces* and *Geosmithia* species. Antonie van Leeuwenhoek, Int J Gen Mol Microbiol 2012;101:403–21.

[12] Wilson DB. Processive and nonprocessive cellulases for biofuel production--lessons from bacterial genomes and structural analysis. Appl Microbiol Biotechnol 2012;93:497–502.

[13] Zuroff TR, Xiques SB, Curtis WR. Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic *Clostridium phytofermentans*/yeast co-culture. Biotechnol Biofuels 2013;6:59.

[14] Ghose TK. Measurement of cellulase activities. Pure Appl Chem 1987;59:257–68.

[15] NREL. National Renewable Energy Laboratory. Biomass research - standard biomass analytical procedures 2013. December 20 2013.

[16] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31:426–8.

[17] Dashtban M, Maki M, Leung KT, Mao C, Qin W. Cellulase activities in biomass conversion: measurement methods and comparison. Crit Rev Biotechnol 2010;30:302–9.

[18] Coward-Kelly G, Aiello-Mazzari C, Kim S, Granda C, Holtzapple M. Suggested improvements to the standard filter paper assay used to measure cellulase activity. Biotechnol Bioeng 2003;82:745–9.

[19] Somogyi M. Notes on sugar determination. J Biol Chem 1952;195:19–23.

[20] Nelson N. A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem 1944;153:375–80.

[21] Sugimoto T, Makita T, Watanabe K, Shoji H. Production of multiple extracellular enzyme activities by novel submerged culture of *Aspergillus kawachii* for ethanol production from raw cassava flour. J Ind Microbiol Biotechnol 2012;39:605–12.

[22] Tsao GT, Xia L, Cao N, Gong CS. Solid-state fermentation with *Aspergillus niger* for Cellobiase Production. Appl Biochem Biotechnol 2000;84-86:743–9.

[23] Van Gool MP, Toth K, Schols HA, Szakacs G, Gruppen H. Performance of hemicellulolytic enzymes in culture supernatants from a wide range of fungi on insoluble wheat straw and corn fiber fractions. Bioresour Technol 2012;114:523–8.

[24] Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. Golden, Colorado: 2008.

[25] Bailey MJ, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 1992;23:257–70.

[26] Dutta T, Sahoo R, Sengupta R, Ray SS, Bhattacharjee A, Ghosh S. Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. J Ind Microbiol Biotechnol 2008;35:275–82.

[27] Eman MF, Hossam SH. Improvement of carboxymethyl cellulase production from *Chaetomium cellulolyticum* NRRL 18756 by mutation and optimization of solid state fermentation. Bangladesh J Bot 2011;40:139–47.

[28] Ng I-S, Li C-W, Chan S-P, Chir J-L, Chen PT, Tong C-G, et al. High-level production of a thermoacidophilic beta-glucosidase from *Penicillium citrinum* YS40-5 by solid-state fermentation with rice bran. Bioresour Technol 2010;101:1310–7.

[29] Shahriarinour M, Wahab MNA, Mohamad R, Mustafa S, Ariff AB. Cyanobacterial biomass as n-supplement to oil palm empty fruit bunch (opefb) fibre for improvement of cellulase production by *Aspergillus terreus* in submerged fermentation. BioRessources 2011;6:1696–706.

[30] Rahnama N, Mamat S, Shah MKU, Ling FH, Aini N, Rahman A, et al. Effect of alkali pretreatment of rice straw on cellulase and xylanase production by local *Trichoderma harzianum* SNRS3 under solid state fermentation. BioRessources 2013;8:2881–96.

[31] Samson R, Yilmaz N, Houbraken J, Spierenburg H, Seifert K, Peterson SW, et al. Phylogeny and nomenclature of the genus *Talaromyces* and taxa accommodated in *Penicillium* subgenus *Biverticillium*. Stud Mycol 2011;70:159–83.

[32] Jabasingh SA. Response surface methodology for the evaluation and comparison of cellulase production by *Aspergillus nidulans* SU04 and *Aspergillus nidulans* MTCC344 cultivated on pretreated sugarcane bagasse. Chem Biochem Eng Q 2011;25:501–11.

[33] Pedersen M, Hollensted M, Lange L, Andersen B. Screening for cellulose and hemicellulose degrading enzymes from the fungal genus *Ulocladium*. Int Biodeterior Biodegradation 2009;63:484–9.

[34] Jørgensen H, Olsson L. Production of cellulases by *Penicillium brasilianum* IBT 20888—Effect of substrate on hydrolytic performance. Enzyme Microb Technol 2006;38:381–90.

[35] Marjamaa K, Toth K, Bromann PA, Szakacs G, Kruus K. Novel *Penicillium* cellulases for total hydrolysis of lignocellulosics. Enzyme Microb Technol 2013;52:358–69.

[36] Peterson R, Nevalainen H. *Trichoderma reesei* RUT-C30--thirty years of strain improvement. Microbiology 2012;158:58–68.

[37] Hawksworth DL, Crous PW, Redhead SA, Reynolds DR, Samson RA, Seifert KA, et al. The amsterdam declaration on fungal nomenclature. IMA Fungus 2011;2:105–12.

[38] Yilmaz N, Houbraken J, Hoekstra ES, Frisvad JC, Visagie CM, Samson RA. Delimitation and characterisation of *Talaromyces purpurogenus* and related species. Persoonia 2012;29:39–54.

[39] Frisvad JC, Yilmaz N, Thrane U, Rasmussen KB, Houbraken J, Samson RA. *Talaromyces atroroseus*, a new species efficiently producing industrially relevant red pigments. PLoS One 2013;8:e84102.

[40] Seifert KA, Samson RA, Dewaard JR, Houbraken J, Lévesque CA, Moncalvo J-M, et al. Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. Proc Natl Acad Sci USA 2007;104:3901–6.

[41] Bidartondo M, Bruns T, Blackwell M, Edwards I, Taylor A, Horton T, et al. Preserving accuracy in GenBank. Science (80- ) 2008;319:1616a.

[42] Wang T, Liu X, Yu Q, Zhang X, Qu Y, Gao P, et al. Directed evolution for engineering pH profile of endoglucanase III from *Trichoderma reesei*. Biomol Eng 2005;22:89–94.

[43] Deng Y, Fong SS. Laboratory evolution and multi-platform genome re-sequencing of the cellulolytic actinobacterium *Thermobifida fusca*. J Biol Chem 2011;286:39958–66.

[44] Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B. Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol Biofuels 2009;2:19.

[45] Herold S, Bischof R, Metz B, Seiboth B, Kubicek CP. Xylanase gene transcription in *Trichoderma reesei* is triggered by different inducers representing different hemicellulosic pentose polymers. Eukaryot Cell 2013;12:390–8.

[46] Häkkinen M, Arvas M, Oja M, Aro N, Penttilä M, Saloheimo M, et al. Re-annotation of the CAZy genes of *Trichoderma reesei* and transcription in the presence of lignocellulosic substrates. Microb Cell Fact 2012;11:134.

[47] Stricker AR, Mach RL, de Graaff LH. Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*). Appl Microbiol Biotechnol 2008;78:211–20.

[48] Furukawa T, Shida Y, Kitagami N, Mori K, Kato M, Kobayashi T, et al. Identification of specific binding sites for XYR1, a transcriptional activator of cellulolytic and xylanolytic genes in *Trichoderma reesei*. Fungal Genet Biol 2009;46:564–74.

[49] Portnoy T, Margeot A, Seidl-Seiboth V, Le Crom S, Ben Chaabane F, Linke R, et al. Differential regulation of the cellulase transcription factors XYR1, ACE2, and ACE1 in *Trichoderma reesei* strains producing high and low levels of cellulase. Eukaryot Cell 2011;10:262–71.

[50] Kunitake E, Tani S, Sumitani J, Kawaguchi T. A novel transcriptional regulator, ClbR, controls the cellobiose- and cellulose-responsive induction of cellulase and xylanase genes regulated by two distinct signaling pathways in *Aspergillus aculeatus*. Appl Microbiol Biotechnol 2013;97:2017–28.

[51] Frisvad JC, Larsen TO, Thrane U, Meijer M, Varga J, Samson RA, et al. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS One 2011;6:e23496.

[52] Guais O, Borderies G, Pichereaux C, Maestracci M, Neugnot V, Rossignol M, et al. Proteomics analysis of "Rovabiot Excel", a secreted protein cocktail from the filamentous fungus *Penicillium funiculosum* grown under industrial process fermentation. J Ind Microbiol Biotechnol 2008;35:1659–68.

[53] Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, et al. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 2007;25:221–31.

[54] Ward OP. Production of recombinant proteins by filamentous fungi. Biotechnol Adv 2012;30:1119–39.

[55] Sørensen A, Lübeck PS, Lübeck M, Teller PJ, Ahring BK. β -Glucosidases from a new *Aspergillus* species can substitute commercial β -glucosidases for saccharification of lignocellulosic biomass. Can J Microbiol 2011;650:638–50.

[56] Sørensen A, Andersen JJ, Ahring BK, Teller PJ, Lübeck M. Screening of carbon sources for beta-glucosidase production by *Aspergillus saccharolyticus*. Int Biodeterior Biodegradation 2014;93:78–83.

[57] Visser EM, Falkoski DL, de Almeida MN, Maitan-Alfenas GP, Guimarães VM. Production and application of an enzyme blend from *Chrysoporthe cubensis* and *Penicillium pinophilum* with potential for hydrolysis of sugarcane bagasse. Bioresour Technol 2013;144:587–94.

[58] Rana V, Eckard AD, Teller P, Ahring BK. On-site enzymes produced from *Trichoderma reesei* RUT-C30 and *Aspergillus saccharolyticus* for hydrolysis of wet exploded corn stover and loblolly pine. Bioresour Technol 2014;154:282–9.

[59] Long C, Ou Y, Guo P, Liu Y, Cui J, Long M, et al. Cellulase production by solid state fermentation using bagasse with *Penicillium decumbens* L-06. Ann Microbiol 2009;59:517–23.

[60] Lee, Sangjoon, Jang Y, Lee YM, Lee J, Lee H, et al. Rice straw-decomposing fungi and their cellulolytic and xylanolytic enzymes. J Microbiol Biotechnol 2011;21:1322–9.

[61] Kang S, Park YS, Lee JS, Hong SI, Kim SW. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresour Technol 2004;91:153–6.

[62] Díaz AB, de Ory I, Caro I, Blandino A. Enhance hydrolytic enzymes production by *Aspergillus awamori* on supplemented grape pomace. Food Bioprod Process 2012;90:72–8.

[63] Sánchez C. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 2009;27:185–94.

[64] Brijwani K, Vadlani P V. Cellulolytic enzymes production via solid-state fermentation: effect of pretreatment methods on physicochemical characteristics of substrate. Enzyme Res 2011;2011:860134.

[65] Bansal N, Tewari R, Soni R, Soni SK. Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. Waste Manag 2012;32:1341–6.

[66] Betini JHA, Michelin M, Peixoto-Nogueira SC, Jorge JA, Terenzi HF, Polizeli MLTM. Xylanases from *Aspergillus niger*, *Aspergillus niveus* and *Aspergillus ochraceus* produced under solid-state fermentation and their application in cellulose pulp bleaching. Bioprocess Biosyst Eng 2009;32:819–24.

[67] Hanif A, Yasmeen A, Rajoka MI. Induction, production, repression, and de-repression of exoglucanase synthesis in *Aspergillus niger*. Bioresour Technol 2004;94:311–9.

[68] Javed MM, Zahoor S, Shafaat S, Mehmooda I, Gul A, Rasheed H, et al. Wheat bran as a brown gold: Nutritious value and its biotechnological applications. African J Microbiol Res 2012;6:724–33.

[69] Kar S, Sona Gauri S, Das A, Jana A, Maity C, Mandal A, et al. Process optimization of xylanase production using cheap solid substrate by *Trichoderma reesei* SAF3 and study on the alteration of behavioral properties of enzyme obtained from SSF and SmF. Bioprocess Biosyst Eng 2013;36:57–68.

[70] Dhillon GS, Oberoi HS, Kaur S, Bansal S, Brar SK. Value-addition of agricultural wastes for augmented cellulase and xylanase production through solid-state tray fermentation employing mixed-culture of fungi. Ind Crops Prod 2011;34:1160–7.

[71] Maeda H, Sano M, Maruyama Y, Tanno T, Akao T, Totsuka Y, et al. Transcriptional analysis of genes for energy catabolism and hydrolytic enzymes in the filamentous fungus *Aspergillus oryzae* using cDNA microarrays and expressed sequence tags. Appl Microbiol Biotechnol 2004;65:74–83.

[72] Sukumaran RK, Singhania RR, Pandey A. Microbial cellulases - production , applications and challenges. J Sci Ind Res 2005;64:832–44.

[73] Fujii T, Hoshino T, Inoue H, Yano S. Taxonomic revision of the cellulose-degrading fungus *Acremonium cellulolyticus* nomen nudum to *Talaromyces* based on phylogenetic analysis. FEMS Microbiol Lett 2013;351:32–41.

[74] Fang X, Yano S, Inoue H, Sawayama S. Lactose enhances cellulase production by the filamentous fungus *Acremonium cellulolyticus*. J Biosci Bioeng 2008;106:115–20.

[75] Xiong H, Turunen O, Pastinen O, Leisola M, von Weymarn N. Improved xylanase production by *Trichoderma reesei* grown on L-arabinose and lactose or D-glucose mixtures. Appl Microbiol Biotechnol 2004;64:353–8.

[76] Pandey A. Solid-state fermentation. Biochem Eng J 2003;13:81–4.

[77] Te Biesebeke R, Ruijter G, Rahardjo YSP, Hoogschagen MJ, Heerikhuisen M, Levin A, et al. Aspergillus oryzae in solid-state and submerged fermentations. Progress report on a multi-disciplinary project. FEMS Yeast Res 2002;2:245–8.

[78] Papagianni M. Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol Adv 2004;22:189–259.

[79] Krishna C. Solid-state fermentation systems-an overview. Crit Rev Biotechnol 2005;25:1–30.

[80] Prévot V, Lopez M, Copinet E, Duchiron F. Comparative performance of commercial and laboratory enzymatic complexes from submerged or solid-state fermentation in lignocellulosic biomass hydrolysis. Bioresour Technol 2013;129:690–3.

[81] Schmidt J, Szakács G, Cenkvári É, Sipocz J, Urbánszki K, Tengerdy RP. Enzyme assisted ensiling of alfalfa with enzymes by solid substrate fermentation. Bioresour Technol 2001;76:207–12.

[82] Machida M, Yamada O, Gomi K. Genomics of *Aspergillus oryzae*: learning from the history of koji mold and exploration of its future. DNA Res 2008;15:173–83.

[83] Ishida H, Hata Y, Ichikawa E, Suginami K, Imayasu S. Regulation of the glucoamylase-encoding gene (glaB), expressed in solid-state culture (koji) of *Aspergillus oryzae*. J Ferment Bioeng 1998;86:301–7.

[84] Te Biesebeke R, Van Biezen N, De Vos W, Van Den Hondel C, Punt P. Different control mechanisms regulate glucoamylase and protease gene transcription in *Aspergillus oryzae* in solid-state and submerged fermentation. Appl Microbiol Biotechnol 2005;67:75–82.

[85] Chen H, He Q. Value-added bioconversion of biomass by solid-state fermentation. J Chem Technol Biotechnol 2012;87:1619–25.

[86] Reactor for sterile solid-state fermentation methods. EP. Patent. WO 1994/EP 0683815 B1.

[87] Solid state fermentation device and products obtained. Fr. Patent. WO 2014/118757 A1.

[88] Bioreactor for fermenting solids. USA. Patent. US 2004/6,797,508 B1.

[89] Directed selective solid-phase culturing of stable microbial mixed populations for the continous preparation of defined enzyme and metabolite mixtures. EP. Patent. WO 2004/113490 A2.

[90] Solid state fermentation. USA. Patent. US 2003/6664095 B1.

[91] Hooge DM, Pierce JL, McBride KW, Rigolin PJ. Meta-analysis of broiler chicken trials using diets with or without allzyme SSF enzyme complex. Int J Poult Sci 2010;9:819–23.

[92] Solid state fermentation (SSF) System. USA. Patent. US 2012/0028345 A1.

[93] Viniegra-González G, Favela-Torres E, Aguilar CN, Rómero-Gomez SDJ, Díaz-Godínez G, Augur C. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochem Eng J 2003;13:157–67.

[94] Nakkeeran E, Gowthaman MK, Umesh-Kumar S, Subramanian R. Techno-economic analysis of processes for *Aspergillus carbonarius* polygalacturonase production. J Biosci Bioeng 2012;113:634–40.

[95] Demir A, Aytar P, Gedikli S, Çabuk A, Arisoy M. Laccase production with submerged and solid state fermentation: benefit and cost analysis. Hacettepe J Biol Chem 2011;39:305–13.

[96] Castilho LR, Polato CM, Baruque EA, Sant Anna Jr GL, Freire DM. Economic analysis of lipase production by *Penicillium restrictum* in solid-state and submerged fermentations. Biochem Eng J 2000;4:239–47.

[97] Ali HKQ, Zulkali MMD. Design aspects of bioreactors for solid-state fermentation: A review. Chem Biochem Eng Q 2011;25:255–66.

[98] Díaz-Godínez G, Soriano-Santos J, Augur C, Viniegra-González G. Exopectinases produced by *Aspergillus niger* in solid-state and submerged fermentation: a comparative study. J Ind Microbiol Biotechnol 2001;26:271–5.

[99] De Souza DF, Giatti Marques de Souza C, Peralta RM. Effect of easily metabolizable sugars in the production of xylanase by *Aspergillus tamarii* in solid-state fermentation. Process Biochem 2001;36:835–8.

[100] Hölker U, Lenz J. Solid-state fermentation--are there any biotechnological advantages? Curr Opin Microbiol 2005;8:301–6.

[101] Rani R, Kumar A, Soccol CR, Pandey A. Recent advances in solid-state fermentation. Biochem Eng J 2009;44:13–8.

[102] Chen H. Modern solid state fermentation. Dordrecht: Springer Netherlands; 2013.

[103] Vaidyanathan S, Macaloney G, Vaughan J, McNeil B, Harvey LM. Monitoring of submerged bioprocesses. Crit Rev Biotechnol 1999;19:277–316.

[104] Krull R, Wucherpfennig T, Esfandabadi ME, Walisko R, Melzer G, Hempel DC, et al. Characterization and control of fungal morphology for improved production performance in biotechnology. J Biotechnol 2013;163:112–23.

[105] El-Enshasy HA. Chapter 9. Filamentous fungal cultures – process characteristics, products, and applications. Bioprocessing for value-added products from renewable ressources new technologies and applications. Amsterdam: Elsevier, 2007, p. 225–61.

[106] Wösten HAB, van Veluw GJ, de Bekker C, Krijgsheld P. Heterogeneity in the mycelium: implications for the use of fungi as cell factories. Biotechnol Lett 2013;35:1155–64.

[107] Sohail M, Siddiqi R, Ahmad A, Khan SA. Cellulase production from *Aspergillus niger* MS82: effect of temperature and pH. N Biotechnol 2009;25:437–41.

[108] Beroviè M, Popovic M. Characterisation of gas mixed bioreactors in submerged citric acid fermentation. Chem Biochem Eng Q 2001;15:65–9.

[109] Chisti Y. Bioreactor design. Basic Biotechnology. Cambridge University Press, Cambridge, 2006, p. 181–200.

[110] Fontana RC, Polidoro TA, da Silveira MM. Comparison of stirred tank and airlift bioreactors in the production of polygalacturonases by *Aspergillus oryzae*. Bioresour Technol 2009;100:4493–8.

[111] Workman M, Andersen MR, Thykaer J. integrated approaches for assessment of cellular performance in industrially relevant filamentous fungi. Ind Biotechnol 2013;9:337–44.

[112] McNeil B, Berry DR, Harvey LM, Grant A, White S. Measurement of autolysis in submerged batch cultures of *Penicillium chrysogenum*. Biotechnol Bioeng 1998;57:297–305.

[113] White S, McIntyre M, Berry DR, McNeil B. The autolysis of industrial filamentous fungi. Crit Rev Biotechnol 2002;22:1–14.

[114] Zhang G, Zhu Y, Wei D, Wang W. Enhanced production of heterologous proteins by the filamentous fungus *Trichoderma reesei* via disruption of the alkaline serine protease SPW combined with a pH control strategy. Plasmid 2014;71:16–22.

[115] De Bekker C, van Veluw GJ, Vinck A, Wiebenga LA, Wösten HAB. Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. Appl Environ Microbiol 2011;77:1263–7.

[116] Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme Microb Technol 2010;46:541–9.

[117] Kalogeris E, Christakopoulos P, Kekos D, Macris B. Studies on the solid-state production of thermostable endoxylanases from *Thermoascus aurantiacus*: Characterization of two isozymes. J Biotechnol 1998;60:155–63.

[118] Kalogeris E, Fountoukides G, Kekos D, Macris BJ. Design of a solid-state bioreactor for thermophilic microorganisms. Bioresour Technol 1999;67:313–5.

[119] Singhvi MS, Adsul MG, Gokhale DV. Comparative production of cellulases by mutants of *Penicillium janthinellum* NCIM 1171 and its application in hydrolysis of Avicel and cellulose. Bioresour Technol 2011;102:6569–72.

[120] Adsul MG, Terwadkar AP, Varma AJ, Gokhale DV. Cellulases from *Penicillium janthinellum* mutants: solid state production and their stability in ionic liquids. BioResources 2009;4:1670–81.

[121] Adsul MG, Bastawde KB, Varma AJ, Gokhale DV. Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production. Bioresour Technol 2007;98:1467–73.

[122] De Almeida MN, Guimarães VM, Bischoff KM, Falkoski DL, Pereira OL, Gonçalves DS, et al. Cellulases and hemicellulases from endophytic *Acremonium* species and its application on sugarcane bagasse hydrolysis. Appl Biochem Biotechnol 2011;165:594–610.

[123] Mahmood RT, Asad MJ, Mehboob N, Mushtaq M, Gulfraz M, Asgher M, et al. Production, purification, and characterization of exoglucanase by *Aspergillus fumigatus*. Appl Biochem Biotechnol 2013;170:895–908.

[124] Saqib AAN, Hassan M, Khan NF, Baig S. Thermostability of crude endoglucanase from *Aspergillus fumigatus* grown under solid state fermentation (SSF) and submerged fermentation (SmF). Process Biochem 2010;45:641–6.

[125] Botella C, Ory I De, Webb C, Cantero D, Blandino A. Hydrolytic enzyme production by *Aspergillus awamori* on grape pomace. Biochem Eng J 2005;26:100–6.

[126] Venegas IM, Fuentes-Hernández J, García-Rivero M, Martínez-Trujillo A. Characteristics of *Aspergillus niger* xylanases produced on rice husk and wheat bran in submerged culture and solid-state fermentation for an applicability proposal. Int J Food Sci Technol 2013;48:1798–807.

[127] Gao J, Weng H, Zhu D, Yuan M, Guan F, Xi Y. Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. Bioresour Technol 2008;99:7623–9.

[128] Narra M, Dixit G, Divecha J, Madamwar D, Shah AR. Production of cellulases by solid state fermentation with *Aspergillus terreus* and enzymatic hydrolysis of mild alkali-treated rice straw. Bioresour Technol 2012;121:355–61.

[129] Pandya JJ, Gupte A. Production of xylanase under solid-state fermentation by *Aspergillus tubingensis* JP-1 and its application. Bioprocess Biosyst Eng 2012;35:769–79.

[130] Shamala TR, Sreekantiah KR. Production of cellulases and d-xylanase by some selected fungal isolates. Enzyme Microb Technol 1986;8:178–82.

[131] Qin Y, He H, Li N, Ling M, Liang Z. Isolation and characterization of a thermostable cellulase-producing *Fusarium chlamydosporum*. World J Microbiol Biotechnol 2010;26:1991–7.

[132] Panagiotou G, Kekos D, Macris BJ, Christakopoulos P. Production of cellulolytic and xylanolytic enzymes by *Fusarium oxysporum* grown on corn stover in solid state fermentation. Ind Crops Prod 2003;18:37–45.

[133] Narang S, Sahai V, Bisaria VS. Optimization of xylanase production by *Melanocarpus albomyces* IIS68 in solid state fermentation using response surface methodology. J Biosci Bioeng 2001;91:425–7.

[134] Li Y, Peng X, Chen H. Comparative characterization of proteins secreted by *Neurospora sitophila* in solid-state and submerged fermentation. J Biosci Bioeng 2013;116:493–8.

[135] Yang SQ, Yan QJ, Jiang ZQ, Li LT, Tian HM, Wang YZ. High-level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid-state fermentation. Bioresour Technol 2006;97:1794–800.

[136] Job J, Sukumaran RK, Jayachandran K. Production of a highly glucose tolerant β-glucosidase by *Paecilomyces variotii* MG3: optimization of fermentation conditions using Plackett–Burman and Box–Behnken experimental designs. World J Microbiol Biotechnol 2010;26:1385–91.

[137] Zhang H, Sang Q. Statistical optimization of cellulases production by *Penicillium chrysogenum* QML-2 under solid-state fermentation and primary application to chitosan hydrolysis. World J Microbiol Biotechnol 2012;28:1163–74.

[138] Muthezhilan R, Ashok R, Jayalakshmi S. Production and optimization of thermostable alkaline xylanase by *Penicillium oxalicum* in solid state fermentation. African J Microbiol Res 2007;1:20–8.

[139] Pothiraj C, Balaji P, Eyini M. Enhanced production of cellulases by various fungal cultures in solid state fermentation of cassava waste. African J Biotechnol 2006;5:1882–5.

[140] Jatinder K, Chadha BS, Saini HS. Optimization of culture conditions for production of cellulases and xylanases by *Scytalidium thermophilum* using Response Surface Methodology. World J Microbiol Biotechnol 2006;22:169–76.

[141] Guerra G, Casado MRG, Arguelles J, Sánchez IMA, Manzano AM, Guzman T. Cellulase Production with sugarcane straw by *Trichoderma citrinoviride* on solid bed. Sugar Technol 2006;8:30–5.

[142] Fadel M. High-level xylanase production from sorghum flour by a newly isolate of *Trichoderma harzianum* cultivated under solid state fermentation. Ann Microbiol 2001;51:61–78.

[143] Liu J, Yang J. Cellulase production by *Trichoderma koningii* AS3.4262 in solid-state fermentation using lignocellulosic waste from the vinegar industry. Food Technol Biotechnol 2007;9862:420–5.

[144] Latifian M, Hamidi-Esfahani Z, Barzegar M. Evaluation of culture conditions for cellulase production by two *Trichoderma reesei* mutants under solid-state fermentation conditions. Bioresour Technol 2007;98:3634–7.

[145] Rocky-Salimi K, Hamidi-Esfahani Z. Evaluation of the effect of particle size, aeration rate and harvest time on the production of cellulase by *Trichoderma reesei* QM9414 using response surface methodology. Food Bioprod Process 2010;88:61–6.

[146] Oberoi HS, Chavan Y, Bansal S, Dhillon GS. Production of cellulases through solid state fermentation using kinnow pulp as a major substrate. Food Bioprocess Technol 2008;3:528–36.

[147] Sun H, Li J, Zhao P, Peng M. Banana peel: A novel substrate for cellulase production under solid-state fermentation. African J Biotechnol 2011;10:17887–90.

[148] Oliveira LA, Porto ALF, Tambourgi EB. Production of xylanase and protease by *Penicillium janthinellum* CRC 87M-115 from different agricultural wastes. Bioresour Technol 2006;97:862–7.

[149] Palma MB, Ferreira Milagres AM, Prata AMR, de Mancilha IM. Influence of aeration and agitation rate on the xylanase activity from *Penicillium janthinellum*. Process Biochem 1996;31:141–5.

[150] Christakopoulos P, Kekos D, Kolisis FN, Macris BJ. Controlling simultaneous production of endoglucanase and beta-glucosidase by *Fusarium oxysporum* in submerged Culture. Biotechnol Lett 1995;17:883–8.

[151] Christov L., Szakacs G, Balakrishnan H. Production, partial characterization and use of fungal cellulase-free xylanases in pulp bleaching. Process Biochem 1999;34:511–7.

[152] Pal A, Khanum F. Identification and optimization of critical medium components using statistical experimental designs for enhanced production of xylanase from *Aspergillus flavus* DFR-6. Food Technol Biotechnol 2011;49:228–36.

[153] Bailey MJ, Poutanen K. Production of xylanolytic enzymes by strains of *Aspergillus*. Appl Microbiol Biotechnol 1989;30:5–10.

[154] Bagga PS, Sandhu DK. Cellulase formation by *Aspergillus nidulans*. J Ferment Technol 1987;65:635–42.

[155] Taneja K, Gupta S, Kuhad RC. Properties and application of a partially purified alkaline xylanase from an alkalophilic fungus *Aspergillus nidulans* KK-99. Bioresour Technol 2002;85:39–42.

[156] Cunha FM, Esperança MN, Zangirolami TC, Badino AC, Farinas CS. Sequential solid-state and submerged cultivation of *Aspergillus niger* on sugarcane bagasse for the production of cellulase. Bioresour Technol 2012;112:270–4.

[157] Min BJ, Park YS, Kang SW, Song YS, Lee JH, Park C, et al. Statistical optimization of medium components for the production of Xylanase by *Aspergillus niger* KK2 in submerged cultivation. Biotechnol Bioprocess Eng 2007;12:302–7.

[158] Matkar K, Chapla D, Divecha J, Nighojkar A, Madamwar D. Production of cellulase by a newly isolated strain of *Aspergillus sydowii* and its optimization under submerged fermentation. Int Biodeterior Biodegradation 2013;78:24–33.

[159] Bakri Y, Masson M, Thonart P. Isolation and identification of two new fungal strains for xylanase production. Appl Biochem Biotechnol 2010;162:1626–34.

[160] Li D-C, Lu M, Li Y-L, Lu J. Purification and characterization of an endocellulase from the thermophilic fungus *Chaetomium thermophilum* CT2. Enzyme Microb Technol 2003;33:932–7.

[161] Miettinen-Oinonen A, Londesborough J, Joutsjoki V, Lantto R, Vehmaanperä J. Three cellulases from *Melanocarpus albomyces* for textile treatment at neutral pH. Enzyme Microb Technol 2004;34:332–41.

[162] Saha BC. Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Process Biochem 2004;39:1871–6.

[163] Romero MD, Aguado J, González L, Ladero M. Cellulase production by *Neurospora crassa* on wheat straw. Enzyme Microb Technol 1999;25:244–50.

[164] Chinedu SN, Okochi VI, Omidiji O. Cellulase production by wild strains of *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma harzianum* grown on waste cellulosic materials. Ife J Sci 2011;13:57–63.

[165] Nwodo SC, Uzoma AO, Thompson NE, Victoria IO. Xylanase production of *Aspergillus niger* and *Penicillium chrysogenum* from ammonia pretreated cellulosic waste. Res J Microbiol 2008;3:246–53.

[166] Haas H, Herfurth E, Stöffler G, Redl B. Purification, characterization and partial amino acid sequences of a xylanase produced by *Penicillium chrysogenum*. Biochim Biophys Acta 1992;1117:279–86.

[167] Camassola M, Dillon AJP. Effect of methylxanthines on production of cellulases by *Penicillium echinulatum*. J Appl Microbiol 2007;102:478–85.

[168] Rapp P, Grote E, Wagner F. Formation and location of 1,4-β-glucanases and 1,4-β-glucosidases from *Penicillium janthinellum*. Appl Environ Microbiol 1981;41:857–66.

[169] Belghith H, Ellouz-Chaabouni S, Gargouri A. Biostoning of denims by *Penicillium occitanis* (Pol6) cellulases. J Biotechnol 2001;89:257–62.

[170] Fang X, Yano S, Inoue H, Sawayama S. Strain improvement of *Acremonium cellulolyticus* for cellulase production by mutation. J Biosci Bioeng 2009;107:256–61.

[171] De Castro AM, de Albuquerque de Carvalho ML, Leite SGF, Pereira N. Cellulases from *Penicillium funiculosum*: production, properties and application to cellulose hydrolysis. J Ind Microbiol Biotechnol 2010;37:151–8.

[172] Brown JA, Coilin SA, Wood TM. Development of a medium for high cellulase, xylanase and β-glucosidase production by a mutant strain (NTG III/6) of the cellulolytic fungus *Penicillium pinophilum*. Enzyme Microb Technol 1989;9:355–60.

[173] De Castro AM, Pedro KCNR, da Cruz JC, Ferreira MC, Leite SGF, Pereira N. *Trichoderma harzianum* IOC-4038: A promising strain for the production of a cellulolytic complex with significant β-glucosidase activity from sugarcane bagasse cellulignin. Appl Biochem Biotechnol 2010;162:2111–22.

[174] Saddler JN, Hogan CM, Louis-Seize G. A comparison between the cellulase systems of *Trichoderma harzianum* E58 and *Trichoderma reesei* C30. Appl Microbiol Biotechnol 1985;22:139–45.

[175] Lejeune R, Baron GV. Effect of agitation on growth and enzyme production of *Trichoderma reesei* in batch fermentation. Appl Microbiol Biotechnol 1995;43:249–58.

[176] Olsson L, Christensen TMIE, Hansen KP, Palmqvist E. Influence of the carbon source on production of cellulases, hemicellulases and pectinases by *Trichoderma reesei* Rut C-30. Enzyme Microb Technol 2003;33:612–9.

[177] Warzywoda M, Ferre V, Pourquie J. Development of a culture medium for large-scale production of cellulolytic enzymes by *Trichoderma reesei*. Biotechnol Bioeng 1983;25:3005–11.

[178] Lan T-Q, Wei D, Yang S-T, Liu X. Enhanced cellulase production by *Trichoderma viride* in a rotating fibrous bed bioreactor. Bioresour Technol 2013;133:175–82.

[179] Adsul M, Ghule J, Singh R, Shaikh H, Bastawde K, Gokhale D, et al. Polysaccharides from bagasse: applications in cellulase and xylanase production. Carbohydr Polym 2004;57:67–72.

[180] Kabel MA, van der Maarel MJEC, Klip G, Voragen AGJ, Schols HA. Standard assays do not predict the efficiency of commercial cellulase preparations towards plant materials. Biotechnol Bioeng 2006;93:56–63.

[181] Lynd LR, Weimer PJ, Zyl WH Van, Isak S. Microbial Cellulose Utilization: Fundamentals and Biotechnology. Microbiol Mol Biol Rev 2002;66:506–77.

[182] Dionisi D, Anderson JA, Aulenta F, McCue A, Paton G. The potential of microbial processes for lignocellulosic biomass conversion to ethanol: a review. J Chem Technol Biotechnol 2015;90:366–83.

[183] Dueñas R, Tengerdy RP, Gutierrez-Correa M. Cellulase production by mixed fungi in solid-substrate fermentation of bagasse. World J Microbiol Biotechnol 1995;11:333–7.

[184] Dwivedi P, Vivekanand V, Pareek N, Sharma A, Singh RP. Co-cultivation of mutant *Penicillium oxalicum* SAU E-3.510 and *Pleurotus ostreatus* for simultaneous biosynthesis of xylanase and laccase under solid-state fermentation. N Biotechnol 2011;28:616–26.

[185] Gutierrez-Correa M, Portal L, Moreno P, Tengerdy RP. Mixed culture solid substrate fermentation of *Trichoderma reesei* with *Aspergillus niger* on sugar cane bagasse. Bioresour Technol 1999;68:173–8.

[186] Gutierrez-Correa M, Tengerdy RP. Production of cellulase on sugar cane bagasse by fungal mixed culture solid substrate fermentation. Biotechnol Lett 1997;19:665–7.

[187] Kolasa M, Ahring BK, Lübeck PS, Lübeck M. Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. Bioresour Technol 2014;169:143–8.

[188] Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresour Technol 2010;101:4851–61.

[189] Park EY, Naruse K, Kato T. One-pot bioethanol production from cellulose by co-culture of *Acremonium cellulolyticus* and *Saccharomyces cerevisiae*. Biotechnol Biofuels 2012;5:64.

# Chapter 5

# Optimization of *Aspergillus carbonarius* ITEM 5010 enzyme production in solid state fermentation

Manuscript in preparation

This chapter concerns studies on optimization of the crude enzyme production by *A. carbonarius*, by altering cultivation parameters in solid state fermentation (SSF). The results are presented in a manuscript form.

# Optimization of *Aspergillus carbonarius* ITEM 5010 enzyme production in solid state fermentation

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## 5.1 Abstract

On-site enzyme production provides an optimal strategy for lowering the enzyme conversion cost in biorefineries, especially the application of lignocellulosic waste for the production of the enzymes would be cost-efficient. Therefore it was analyzed whether *Aspergillus carbonarius* enzyme production could be optimized using different lignocellulosic waste media and three cultivation times 5, 7 and 10 days at three temperatures 25, 30 and 35 °C. The resulting crude enzymes were evaluated by hydrolysis of wheat straw, while also analyzing for xylanase, β-glucosidase and avicelase activities. The hypercellulase producing mutant *Trichoderma reesei* Rut-C30 was used as a reference strain. Six different media were analyzed, wherein the major components were wheat bran (WB), garden and park waste (GPW), municipal organic solid waste, sphagnum peat and lactose. The best medium for enzyme production was the GPW with added nitrogen source (GPW/N), which resulted in 5.9 g/L glucose release after 5 days cultivation at 30 °C. This was, however, not the medium that gave highest β-glucosidase, xylanase or avicelase activities. For these activities, the best media were WB + lactose or WB + sphagnum peat. A prolonged cultivation time resulted in higher β-glucosidase, with a maximum of 17.04 U/mL reached after 10 days cultivation. It was also apparent that optimal enzyme production in all media was achieved by cultivation at 30 °C. For *T. reesei* Rut-C30 the best medium was also GPW/N which resulted in highest activities for all enzymes analyzed and also the highest glucose release in wheat straw hydrolysis (6.1 g/L). The high activities observed in the GPW/N medium was found to be strongly influenced by the additional nitrogen source, urea and $(NH_4)_2SO_4$, as low activities were obtained when these two nitrogen sources were removed. When cultivation is kept at 5 days the GPW/N appears as the optimal medium for both *A. carbonarius* and *T. reesei* Rut-C30, media based on GPW would therefore be relevant for on-site enzyme production. The WB/SP medium could though be viewed as a good alternative for *A. carbonarius* due to the high β-glucosidase achieved after 10 days cultivation.

*Keywords*: *Aspergillus carbonarius*; SSF; cellulase production; Crude enzymes; Hydrolysis synergy; Ascomycetes; SSF

## 5.2 Introduction

Lignocellulose the main component of plant biomass is a valuable resource for production of biofuels and high value chemicals in biorefinery settings. However, to fully utilize the biomass an enzymatic conversion is required. The enzymes required for lignocellulose conversion are often derived from filamentous fungi belonging to the Ascomycete phylum. Among the known industrial producers are *Aspergillus niger*, *A. oryzae*, *Penicillium funiculosum*, *Trichoderma longibrachiatum*, *T. koningii*, *T. reesei* and *T. viride* [1–5]. The enzymes required for conversion of lignocellulose include the concerted action of cellulases, endo-1,4-β-D-glucanase, exo-β-1,4-glucan cellobiohydrolase (CBH) and β-1,4-glucosidase, as well as the hemicellulases (e.g. exo-1,4-β-xylosidase and endo-1,4-β-xylanase) [6]. Furthermore, the accessory enzyme lytic polysaccharide monooxygenases (LPMO) assigned AA9 in the CAZy database is now a standard addition to commercial cellulase products [7][8]. Despite the fact that commercial preparations are becoming more efficient they are often expensive and decrease the cost efficiency of biorefineries [9]. The cost of enzymes can be reduced through several approaches either by improving the activity of the individual enzymes or by using tailored blends whereby an optimal ratio is obtained increasing efficiency and decreasing the required amount. A more basic approach for decreasing the cost is to improve the production strategy of the lignocellulolytic enzymes to increase yield. Industrial production of lignocellulolytic enzymes is primarily performed in submerged fermentation (SmF), which is an easily scalable approach for industrial enzyme production. It has, however, been shown that there is a promising gain in enzyme yield from using solid state fermentation (SSF). SSF resembles the natural habitat of most filamentous fungi, and should therefore yield higher enzyme titers [10]. It is also possible to apply greater amounts of insoluble substrates such as lignocellulosic waste from agriculture or food industries in SSF. The application of waste streams as substrate in the production of cellulase enzymes would significantly lower the production expenses and thereby decrease the cost of enzymes. Agricultural waste products and slipstream waste from biorefineries have been studied as resources for designing novel low-cost production media [11]. Recently an on-site separate enzyme production of cellulases from *T. reesei* and β-glucosidases from *A. saccharolyticus* was applied using corn stover, corn steep liquor, corn mash and wheat bran (WB) as cultivation substrates [9]. To scale up such an approach it is essential to determine the optimal growth conditions for highest yield of the lignocellulolytic enzymes. In a scaled up process it would be advantageous to apply a waste stream either from the biorefinery, agriculture or elsewhere for the production of on-site enzymes.

This study aims at elucidating optimal conditions in SSF for cellulase production by *T. reesei* Rut-C30 and *Aspergillus carbonarius*, when applying novel waste substrates such as garden and park waste (GPW) and municipal organic waste (MW). These were compared with substrates based on wheat bran which is well-

known as an enzyme inducing substrate. The waste materials were obtained from a Danish company, Solum A/S that collects garden-park and municipal organic waste for different applications. The GPW is composted and sold as soil improving products whereas MW is turned into valuable biogas and nutrient rich compost [12]. However, both waste types represent very low cost materials and novel uses of these materials are highly relevant compared to the current use. The two fungal isolates were chosen based on their known cellulase production [9,13]. The media are analyzed for their application as a substrate in both on-site enzyme production and for a potential consolidated bioprocess setting. Six media were compared for the two fungal strains by analysis of the produced enzyme activities. Furthermore, an appraisal of the produced crude enzymes potential was made, by analyzing hydrolysis of pretreated wheat straw.

## 5.3 Methods

### 5.3.1 *Fungal strains*

This comparative study applied the two strains *Aspergillus carbonarius* (ITEM 5010) and *Trichoderma reesei* Rut-C30 (ATCC 56765). The strains were maintained as live cultures grown on V8 agar and stored at 4 °C.

### 5.3.2 *Preparation of substrates*

Lignocellulosic waste containing branches, leaves and grass from gardens and parks (non-composted) was provided by Solum A/S. It was dried at 90 °C for 48h and autoclaved at 121 °C for 15 min. The dry garden park waste (GPW) was shredded using a SM 2000 cutting mill (Retsch, Germany) and filtered through a mesh of 5 mm. Solid municipal organic waste, also provided by Solum A/S, was sorted to remove larger pieces of plastic and autoclaved at 121 °C for 60 min before drying at 90 °C for 48 h. The dry municipal waste (MW) was milled in a coffee mill and filtered through a mesh of 5 mm. Compositional analysis of both GPW and MW was made using strong acid hydrolysis, 72 % for 1 hour. Nitrogen content based on the Kjehldahl method [14] was determined and protein content was calculated by 6.25 x nitrogen content. The resulting composition of both substrates is shown in Table 5.1. Furthermore, steam-exploded acid pretreated wheat straw (kindly donated by Biogasol A/S, Denmark), contained 22 % total solids/g and the composition is shown in Table 5.1.

**Table 5.1** Composition of acid hydrolyzed GPW, MW and Biogasol pretreated wheat straw, component amounts presented in % (w/w).

| Structural component | Acid hydrolyzed GPW (%) | Acid hydrolyzed MW (%) | Pretreated wheat straw % DM |
|---|---|---|---|
| Cellulose/glucose | 20.5 | 23.8 | 36 |
| Xylan | 10.5 | 4.8 | 16.6 |
| Arabinan | 1.1 | - | 1.5 |

| Structural component | Acid hydrolyzed GPW (%) | Acid hydrolyzed MW (%) | Pretreated wheat straw % DM |
|---|---|---|---|
| Klason lignin | 19.6 | 25.8 (acid insoluble) | 22.1 |
| Ash | 15.7 | 4.4 | 1.3 |
| Protein | 1.9 | 5.3 | - |
| Residual | 30.7 | 35.9 | 22.5 |
| **Total** | **100** | **100** | **100** |

### 5.3.3 *Spore suspensions*

The fungi were grown on minimal medium plates (10 g/L glucose, 10 mM $NaNO_3$, 20 g/L agar and 20 mL/L of trace elements: 0.4 g/L $CuSO_4 \cdot 5H_2O$, 0.8 g/L $FeSO_4 \cdot 7H_2O$, 8 g/L $ZnSO_4 \cdot 7H_2O$, 0.8 g/L $MnSO_4 \cdot 7H_2O$, 0.04 g/L $Na_2B_4O_7 \cdot 10H_2O$ and $Na_2MoO_4 \cdot 2H_2O$). Spores were harvested from 7 day old plate cultures by addition of sterile Milli-Q water with 0.1 % Tween-80 (Sigma Aldrich). Spore suspensions were filtered through Miracloth (Merck), followed by spore counting using a Bürker Türk counting chamber.

### 5.3.4 *Solid state fermentation*

Six types of solid media were prepared containing different carbon and nitrogen sources aiming at a 10:1 ratio: 1) wheat bran + lactose (WB/L), 2) wheat bran + lactose + nitrogen (WB/LN), 3) wheat bran + sphagnum peat (WB/SP), 4) GPW, 5) GPW + nitrogen (GPW/N), 6) GPW + MW (GPW/MW). A liquid medium based on Mandels and Weber [15]: ($KH_2PO_4$ (2 g/L), $MgSO_4 \cdot 7\ H_2O$ (0.3 g/L), $CaCl_2$ (0.3 g/L) and 1 mL/L of trace metals (pH 7.0)) was added to reach 30 % solids. The dry MW was used as a low-cost alternative nitrogen supplement for the GPW. The composition of each medium pr. 1 kg is shown in Table 5.2.

**Table 5.2** Composition pr. 1 kg of medium for the six solid media: WB/L, WB/LN, WB/SP, GPW, GPW/N and GPW/MW.

| | WB/L | WB/LN | WB/SP | GPW | GPW/N | GPW/MW |
|---|---|---|---|---|---|---|
| Wheat bran (WB) | 190.0 g | 190.0 g | 238.0 g | - | - | - |
| Lactose (L) | 111.0 g | 111.0 g | - | - | - | - |
| Mandels and Weber liquid medium | 699.0 g | 699.0 g | - | 700.0 g | 700.0 g | 700.0 g |
| $(NH_4)_2SO_4$ (dissolved in the liquid medium) | - | 3.5 g | - | - | 3.5 g | - |
| Urea (dissolved in the liquid medium) | - | 0.2 g | - | - | 0.2 g | - |
| Sphagnum peat (SP) | - | - | 143.0 g | - | - | - |
| Garden and park waste (GPW) | - | - | - | 300.0 g | 300.0 g | 193.0 g |
| Municipal waste (MW) | - | - | - | - | - | 107.0 g |
| Water | - | - | 619.0 g | - | - | - |

The dry substrates and liquid medium were combined in 250 mL Erlenmeyer flasks and closed with wadding and gaze. For WB/L and WB/LN a total of 26.6 g medium was added to a 250 mL shake flask, and for WB/SP it was 43 g pr. shake flask. A total of 33.3 g of GPW and GPW/N was used pr. shake flask and for GPW/MW it was 38.3 g applied in each of the cultivations. All shake flasks with medium were autoclaved at 121 °C for 15 min. Each flask was inoculated with a concentration of 2 x $10^6$ spores/mL spore suspension for *A. carbonarius* and *T. reesei* Rut-C30 and incubated stationary for 5, 7 and 10 days at 25, 30 and 35 °C. Flasks were shaken by hand twice a day for equal distribution of medium and spores. All growth experiments were performed in duplicates.

### 5.3.5 *Extraction of enzymes*

Enzymes were harvested from the solid state growth by addition of 50 mL of sterile Milli-Q water and extracting overnight at 4-5 °C with shaking at 180 rpm. The liquid fraction was filtered through Miracloth and centrifuged at 4 °C for 30 minutes at 10,000 x G. The resulting supernatant was transferred to fresh 50 mL falcon tubes. The cleared and filtered supernatants were stored at -20 °C prior to use in the enzyme assays.

### 5.3.6 *Enzyme assays*

β-glucosidase activity was determined using 5 mM para-nitrophenyl-β-D-glucopyranoside (pNPG) (Sigma Aldrich) in 50 mM sodium citrate (pH 4.8) as the substrate. The assay was carried out in a microtiter-plate format according to [16]. A 10 µL volume of sample supernatant was added to 100 µL substrate in 1.5 mL Eppendorf tubes, and incubated in a Thermomixer® comfort (Eppendorf) at 50 °C for 15 min. At the end of the reaction 60 µL of the reaction volume was transferred to a microtiter plate already containing 100 µL 1 M $Na_2CO_3$ for termination of the reaction. Absorbance at 405 nm was measured in a plate reader (BioTek, EL800). Para-nitrophenol (pNP) was used for preparation of a standard curve. One unit (U) of enzyme activity was defined as the volume of enzyme needed to hydrolyze 1 µmol of pNPG in 1 min.

Avicelase activity was determined using a 1 % Avicel solution in a hydrolysis assay where total released glucose was measured after 24 hours. The assay was carried out using 500 µL 1 % Avicel PH-101 (Sigma-Aldrich) in 10 mM sodium acetate, pH 5.0, mixed with 250 µL crude enzyme extract and 250 µL 10 mM sodium acetate pH 5.0 and incubated at 50 °C, 1400 rpm (Eppendorf Thermomixer comfort) for 24 h. The reactions were stopped by boiling them at 100 °C for 10 min, and added 45 µL 10 % sulfuric acid before spinning down the solids at 16,000 x G for 10 min. The supernatant was collected for analysis of total amount glucose released by high performance liquid chromatography (HPLC) 1100 series (Agilent) equipped with an Aminex HPX-87H column (Biorad).

Xylanase activity was determined using a solution of 1 % (w/v) birchwood xylan, dissolved in 50 mM sodium citrate, pH 5.0. Eppendorf tubes (1.5 mL) were prepared with 225 µL substrate, and preheated to 50 °C for 3 min before addition of 25 µL enzyme supernatant. The reaction was run for 30 min at 50 °C and stopped by adding 250 µL of NaOH (1 M) and 500 µL of PAHBAH (0.1 M p-hydroxybenzoic acid hydrazine, 0.2 M NaK-tartrate and 0.5 M NaOH). The tubes were then transferred immediately to 100 °C and boiled for 10 min. Finally tubes were placed on ice for 10 min, before centrifuging at 16,000 rpm for 5 min. Supernatant was transferred to a microtiter plate and absorbance was measured at 405 nm. A standard curve of different xylose concentrations reaction with PAHBAH was made and used for calculation of produced xylose (µmol) pr. min. for 1 mL of crude enzyme (U/mL).

### 5.3.7 *Wheat straw hydrolysis*

Supernatants from the cultivations were analyzed for their application potential with regards to hydrolysis of wheat straw. Pretreated wheat straw (Biogasol A/S, Denmark) was applied at 5 % total solids in the reactions, the cellulose content of the wheat straw was 36 % and the amount of total solids 22 % as seen in Table 5.1. The assay was set up to run with 600 µL supernatant added to 5 % wheat straw in combination with 700 µL 0.1 M succinic acid buffer, pH 5.0 and incubated at 50 °C, 1400 rpm (Eppendorf Thermomixer comfort), for 2 days. After incubation the samples were boiled at 100 °C for 10 min, mixed with 45 µL of 10 % sulfuric acid and centrifuged at 16,000 x G, for 10 min. The supernatants were then analyzed for released sugars using Agilent 1100 series HPLC system equipped with an Aminex HPX-87H column (Biorad).

## 5.4 Results and discussion

### 5.4.1 β-*glucosidase*

Crude enzyme harvest after three different durations of cultivation of *A. carbonarius* and *T. reesei* clearly shows the influence of time on enzyme productivity/activity. From Fig. 5.1 it is apparent that by incubation of *A. carbonarius* for 10 days at 30 °C in WB/SP medium, the highest activity of β-glucosidase 17.04 U/mL could be achieved. The temperature significantly influenced the activities since there was a markedly lower activity after 10 days in WB/SP at 25 °C and 35 °C corresponding to 14.40 and 10.87 U/mL, respectively. In general, the WB/SP medium induced the highest activities of β-glucosidase, compared to the GPW medium. Previously reported activity for *A. carbonarius* cultivated in similar conditions in WB/SP for 10 days at 25 °C by Kolasa et al. was approximately 20 U/mL [13], compared to 14.40 U/mL observed in this study. The only difference between the two setups were the amount of water used to extract the crude enzymes, 50 mL used in this study and 100 mL used in [13]. In this study the activity achieved at 25 °C after 10 days, 14.40

U/mL was observed already after 7 days of cultivation at 30 °C. For both 25 and 30 °C there was a positive effect on activity of β-glucosidase by prolonged cultivation compared to at 35 °C where the activity declined from 12.40 after 7 days to 10.87 U/mL after 10 days. Declining activity at 35 °C may be an effect of increased evaporation of the water content in the medium. It could also have been an effect of decreased growth due to the high temperature, or that proteolysis of the produced enzymes occur faster at this temperature.

Interestingly the activity obtained for cultivation in GPW/N for 10 days decrease drastically at both 30 and 35 °C. This may be a result of the medium drying out at the high temperatures during a prolonged incubation time. Since the GPW/N was dried thoroughly before being applied it contained less water compared to the WB/SP, which was not dried prior to use. However, the duration of incubation did have a positive influence on the produced β-glucosidase activity as observed by the rise in activity from 7.29 U/mL after 5 days at 25 °C to 8.06 U/mL at 7 days concluding in the highest activity of 9.30 U/mL after 10 days. The effect of cultivation time on the amount of β-glucosidase produced was most apparent for the WB/SP medium, which could be due to the lower amount of available water in the GPW/N medium.

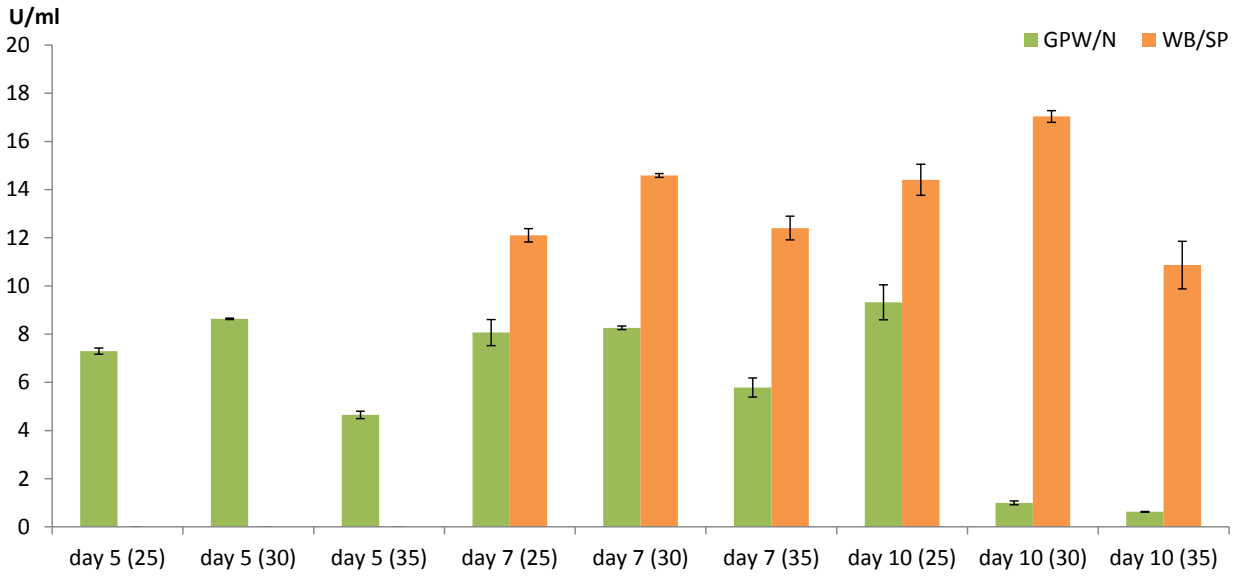


**Figure 5.1** Activity of β-glucosidase for *A. carbonarius* after cultivation in GPW/N (green) for 5, 7 and 10 days and WB/SP medium (orange) for 7 and 10 days at 25, 30 and 35 °C. Error bars denote standard deviation (SD) (N = 2).

The β-glucosidase activity seen for cultivation of *T. reesei* Rut-C30 was, as expected, much lower than *A. carbonarius*, as can be seen in Fig. 5.2 where the highest activity observed was 2.22 U/mL for 10 days cultivation in GPW medium at 25 °C. For the GPW medium it is apparent that the most activity could be obtained from cultivation at 25 °C independent of the cultivation duration. Increasing temperature had a

significantly negative impact on the activity. This was most obvious at 35 °C where activity dropped to 1.1 U/mL. Again it may be a result of the available water content decreasing faster at the increased temperature. For *T. reesei* Rut-C30 the effect of prolonged cultivation on the activity of β-glucosidase could only be observed for WB/SP medium at 30 °C where activity increased from 1.27 U/mL to 1.7 U/mL at 10 days. The overall low level of activity was expected due to the fact that most of *T. reesei* Rut-C30 β-glucosidase is either intra-cellular or membrane bound [17].

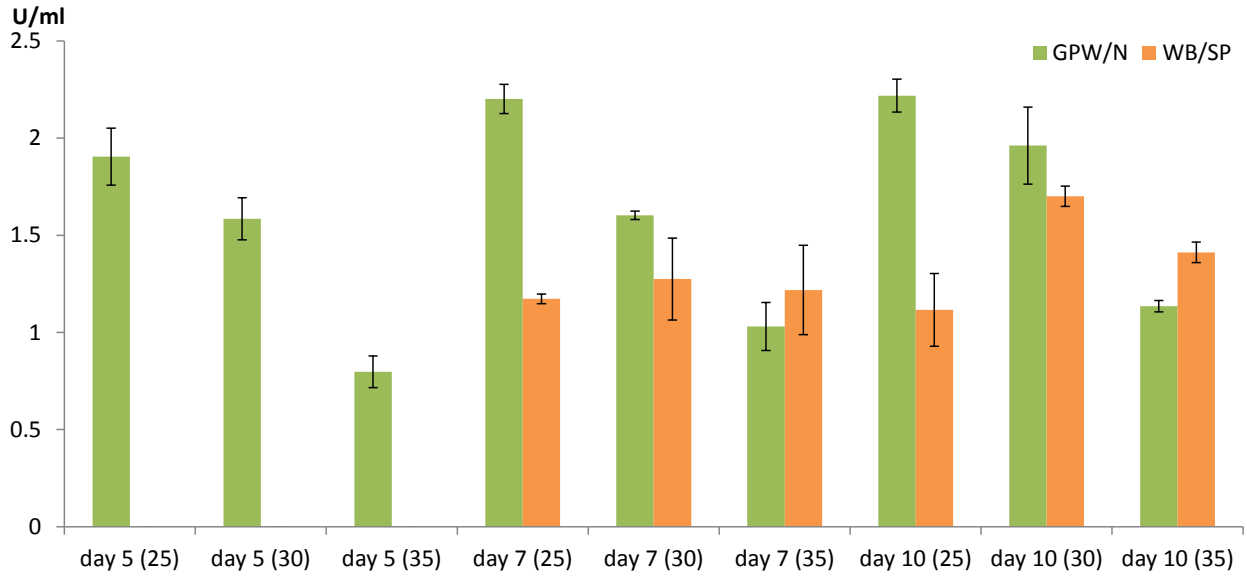


**Figure 5.2** Activity of β-glucosidase in *T. reesei* Rut-C30 crude enzymes after cultivation in GPW/N (green) for 5, 7 and 10 days and WB/SP medium (orange) for 7 and 10 days all at 25, 30 and 35 °C. Error bars denote SD (N = 2).

### 5.3.2 β-*xylanase*

The xylanase activity was measured for the cultivations at 5 days in the three media WB/L, GPW/MW and GPW/N. As seen in Fig. 5.3 (A), the highest xylanase activity for *A. carbonarius* was observed after cultivation in the WB/L medium at 30 °C corresponding to 523.5 U/mL. In general the xylanase activity was highest at 30 °C for all three media. This correlates well with previous studies of Australian isolates of *A. carbonarius* growth rate, which were shown to be highest at 30 °C and with 0.965 $a_w$ [18]. The observed activity in our study is though higher than previous findings for *A. carbonarius* (KLU-93) of 0.149 U/mL, which was obtained from 4 days cultivation under similar conditions using WB and a growth temperature of 27 °C [19]. The large difference in activity may be a result of their usage of p-nitrophenyl β-D-xylopyranoside (pNP-Xyl) as well as a lower reaction temperature of 37 °C in the enzyme assay. Another isolate of *A. carbonarius* (CBS 111.26) has been analyzed by Meijer et al. [20], and shown to have a relatively low specific xylanase activity of 0.15 µmol/min/mg protein using pNP-Xyl. Interestingly, in this

study cultivation of *A. carbonarius* in GPW/MW and GPW/N medium also resulted in activities of 254.0 U/ml and 292.6 U/ml, respectively. The activities are relatively high in comparison with previous results obtained from SmF as it was recently reported that an average xylanase activity of 20 fungal isolates was 117 U/mL [11]. Both GPW/MW and GPW/N are based on cheap wastes and could be applied as efficient alternative production media. In contrast to the observed decreasing β-glucosidase activity in crude enzymes from 35 °C there seem to be an increase of β-xylanase from WB/L and the GPW/N medium, compared to 25 °C.

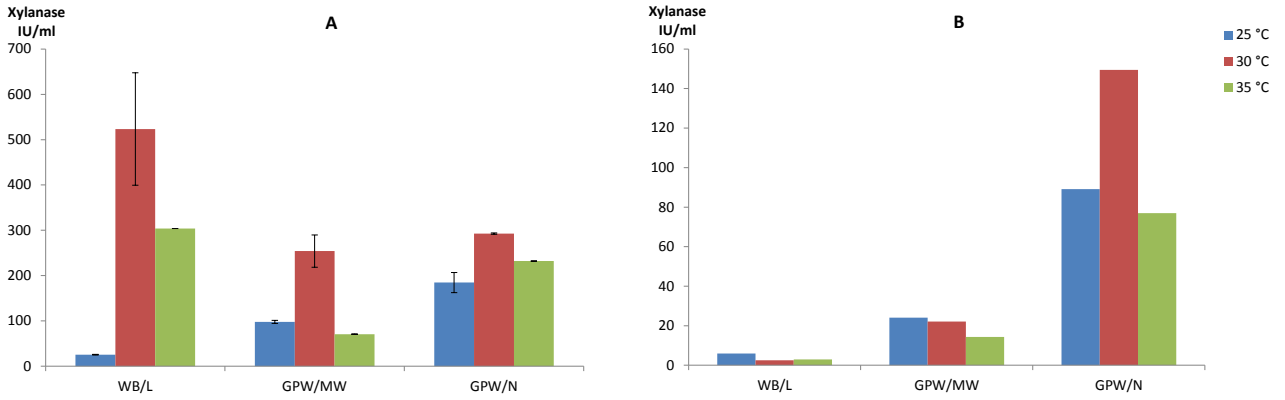


**Figure 5.3** Xylanase activity for crude enzymes after 5 day cultivations in WB/L, GPW/MW and GPW/N at 25 (blue), 30 (red) and 35 °C (green) for A) *A. carbonarius* and B) *T. reesei* Rut-C30. Error bars show SD (N = 2).

The highest β-xylanase of *T. reesei* was also shown to be from cultivation at 30 °C, as seen in Fig. 5.3 (B). However, the optimal medium was GPW, which resulted in higher activities at all cultivation temperatures compared to WB/L and GPW/MW. This indicates that residual sugars in the MW and the WB/L may have a catabolite repressing effect on the expression of xylanase, and that the cellulose/xylan content in GPW/N induces the Xyr1 regulator of *T. reesei*. The activator Xyr1 has been shown to have higher transcript levels when there is no carbon catabolite repression (CCR), and lactose has not been found to stimulate transcription of Xyr1 [21]. However, significantly higher levels of *xyn1* (xylanase 1) transcripts were found for growth on lactose compared to glucose, but the levels were not nearly as high as the ones attained for xylose as carbon source [21]. This indicates that the available sugars in WB may induce CCR resulting in cancellation of lactose induction. The highest activity observed for *T. reesei* Rut-C30 in this study was 149.5 U/mL from growth in GPW/N at 30 °C, which is somewhat lower than 233 U/mL reported for a *T. reesei* (CL-847) strain grown on 6 % lactose [22].

### 5.3.3 *Avicelase*

The influence of medium and cultivation time on the activity of crude enzymes towards crystalline cellulose was analyzed by hydrolysis of Avicel. The Avicel hydrolysis potential of *A. carbonarius* appeared optimal in WB/L after 5 days of cultivation at 25 °C. This yielded a glucose release of 1.0 ± 0.2 g/L as seen in Fig. 5.4, compared to GPW/MW at 0.48 ± 0.2 g/L and GPW/N at 0.43 ± 0.01 g/L. For the WB/L medium cultivation at all temperatures yielded higher activities than the other two media. The GPW/N and GPW/MW resulted in a similar activity at all three temperatures, however with a slight decrease for GPW/N at 35 °C down to 0.32 ± 0.01 g/L glucose.

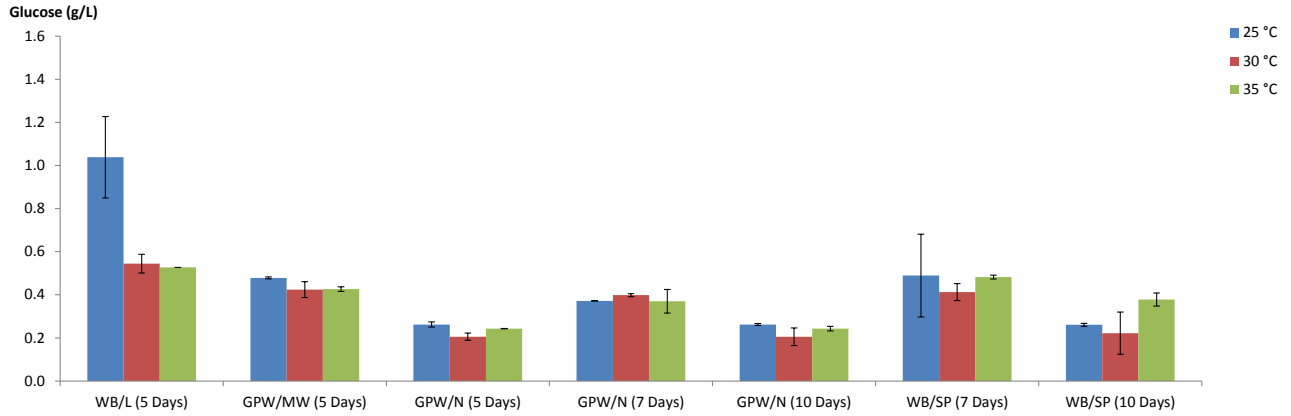


**Figure 5.4** Glucose release (g/L) in Avicel hydrolysis, obtained for *A. carbonarius* from cultivations in: WB/L, GPW/MW, GPW/N and WB/SP, after 5, 7 and 10 days at 25 (blue), 30 (red) and 35°C (green). Error bars denote SD (N = 2).

The effect of prolonged cultivation was analyzed for the GPW/N medium and a modified version of WB/L where lactose was substituted for SP. There was no increase in the Avicel conversion potential of the crude enzyme by increasing the cultivation time. Indicating that the level of exoglucanase produced does not increase by prolonging cultivation time, and that a maximum level is reached already after 5 days. This is, however, relative to the medium applied in the current study. A time course study by Hanif et al. [23], of *A. niger* exoglucanase production showed that the activity reaches a plateau at 5 days of cultivation on α-cellulose and wheat bran. Furthermore, Hanif et al. [23], observed that the optimal nitrogen source for induction of exoglucanase productivity was corn steep liquor followed by sodium glutamate and ammonium sulfate, which was also used as a supplement for GPW/N medium in this study. From the current study it could be observed that WB/SP medium did not result in an increased glucose release compared to GPW/N medium. In a previous study by Kolasa et al. [13], where *A. carbonarius* was also cultivated on WB/SP for 10 days the resulting crude enzyme applied for hydrolysis of Avicel (assay time 2 h) was shown to release only 0.022 g/L glucose. This lower amount may be a result of decreased assay temperature of 37 °C and time 2 h, compared to this study where the assay was performed at 50 °C and for 24 h.

For *T. reesei* the glucose release from Avicel was as expected much higher than for *A. carbonarius*, with the highest activity of 1.49 g/L glucose from crude enzymes of GPW/N cultivation for 5 days at 30 °C (Fig. 5.5). Also for cultivation in WB/L medium a high glucose release of 1.32 g/L was obtained at 25 °C, after 5 days. However, for the GPW/MW medium the lowest activity was seen with an apparent decline of activity for increased temperature in cultivation. It was in general observed that cultivation at 35 °C resulted in lower activity for all media.

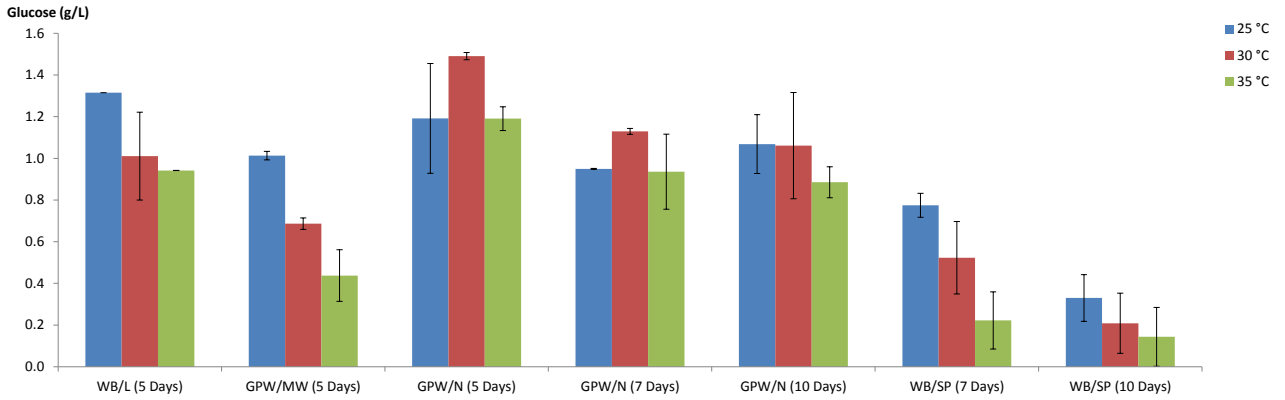


**Figure 5.5** Glucose release (g/L) in Avicel hydrolysis, obtained for *T. reesei* Rut-C30 cultivated in: WB/L, GPW/MW, GPW/N and WB/SP, after 5, 7 and 10 days at 25 (blue), 30 (red) and 35°C (green). Error bars denote SD (N = 2).

The influence of cultivation time on the production of avicelase activity was analyzed for two media GPW/N and WB/SP. Glucose release declines for crude enzymes obtained from GPW/N after incubation for 7 days at 30 °C, to 1.13 g/L glucose. There is not, however, a large decline in activity after 10 days of incubation at 30 °C indicated by the glucose release of 1.06 g/L. Cultivation at 35 °C resulted in the lower activity as seen for 7 days in GPW/N 0.94 g/L and for 10 days 0.89 g/L glucose. In general the lowest activity was observed after cultivation in WB/SP for 7 days at 35 °C resulting in 0.22 g/L glucose and for all temperatures after 10 days cultivation with an average glucose release of 0.23 g/L. Previously Kolasa et al., showed that *T. reesei* Rut-C30 crude enzymes after growth in the same type of WB/SP medium at 25 °C for 10 days could release 0.14 g/L glucose in Avicel hydrolysis [13]. This value is lower than observed in the current study, 0.33 g/L glucose release (Fig. 5.5), and may again be a result of longer duration of the assay (24h) as well as an increased assay temperature of 50 °C applied in this study. Rocky-Salimi et al. [24], have shown that for cultivation of *T. reesei* QM9414 on rice bran, the maximal activity of avicelase 99.7 U/g dry weight substrate could be achieved after 4 days.

### 5.4.2 *Wheat straw hydrolysis*

The application potential of the crude enzymes was also analyzed for hydrolysis efficiency of pretreated wheat straw. The crude enzymes obtained from cultivation of *A. carbonarius* in GPW/N medium resulted in the most efficient lignocellulose hydrolysis as seen in Fig. 5.6. This was especially seen for the crude extracts from 5 days cultivation at 25 and 30 °C, which resulted in 5.84 and 5.90 g/L glucose release, respectively. From 35 °C cultivations in GPW/N the activity in the crude enzymes was decreased resulting in a lower glucose release of 4.88 g/L. Least efficiency in wheat straw hydrolysis was obtained from crude enzymes of cultivation in WB/L medium, where 4.5 g/L glucose was achieved for growth at 30 °C compared to 2.5 and 3.0 g/L at 25 and 35 °C, respectively. Crude enzymes from GPW/MW showed an average glucose release at all temperatures of 4.36 g/L and achieved an overall better result than WB/L. Although it was found that WB/L resulted in high avicelase and xylanase activitites, these did not correlate to a high glucose release in wheat straw hydrolysis. This has been corroborated by Kabel et al. [25], that also showed the missing correlation between standard assays and efficiency towards plant materials. It was therefore apparent that the highest wheat straw hydrolysis efficiency, 28.94 %, could be achieved from GPW/N medium (Fig. 5.6, B). The glucose release of 5.90 g/L from the GPW/N crude enzymes was achieved after only 5 days cultivation relative to 7.5 g/L, which was achieved by Kolasa et al. [13], after cultivation in WB/SP medium for 10 days. Therefore the GPW/N medium may provide a more economical cultivation process, as similar efficiencies of crude enzymes can be reached faster.

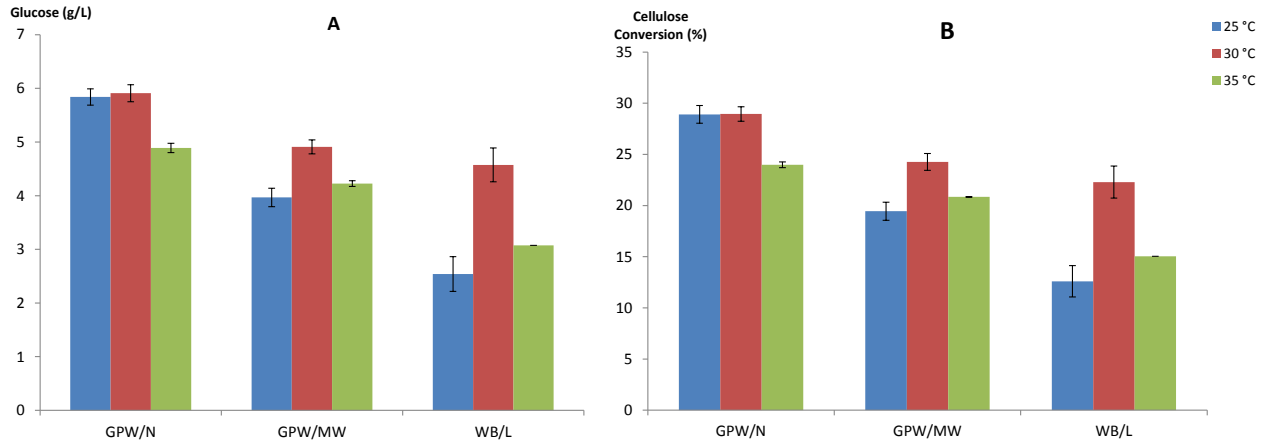


**Figure 5.6** A) Glucose release in hydrolysis of 5 % wheat straw, for *A. carbonarius* enzymes after 5 days cultivation in GPW/N, WB/L and WB/L at 25, 30 and 35 °C. B) Percentage of cellulose conversion. Error bars denote SD (N = 2).

For *T. reesei* Rut-C30 it was also apparent that there was a markedly better glucose release obtained from the GPW/N crude enzymes. Again the optimal temperatures were 25 and 30 °C, which resulted in 5.5 and 6.1 g/L glucose, respectively (Fig. 5.7). Furthermore, it was apparent that at 35 °C the activity decreases

resulting in 4.3 g/L released. WB/L medium led to the lowest glucose release averaging at 1.99 g/L glucose, which was even lower than what was observed for *A. carbonarius*. Also the GPW/MW resulted in much lower glucose release than what was observed for *A. carbonarius* with a difference of 2.1 g/L glucose. Previously *T. reesei* Rut-C30 was shown to produce a very efficient crude enzyme extract when cultivated on WB/SP, which resulted in 12 g/L glucose release [13]. Another study by Rana et al. [9], of *T. reesei* Rut-C30 where the enzyme loading was 5 and 15 filter paper units pr. gram cellulose combined with 10 and 30 β-glucosidase units (from *A. saccharolyticus*) in hydrolysis of wet exploded corn stover and loblolly pine resulted in an efficiency of 58 % and 80 %, respectively.

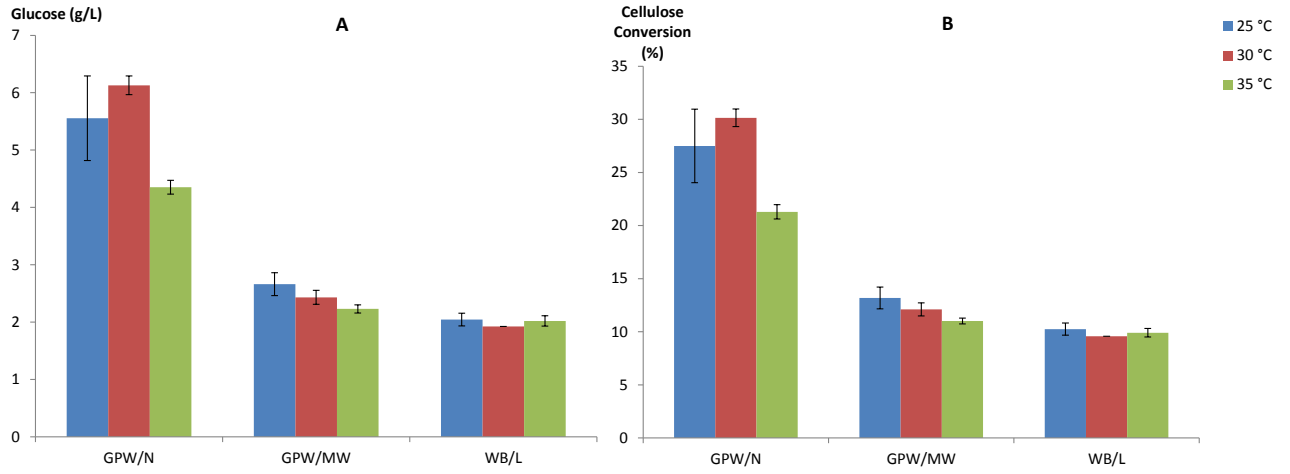


**Figure 5.7** A) Glucose release in hydrolysis of 5 % wheat straw, for *T. reesei* Rut-C30 enzymes after 5 days cultivation in GPW/N, GPW/MW and WB/L at 25, 30 and 35 °C. B) Percentage of cellulose conversion. Error bars denote SD (N = 2).

In this study it was further analyzed whether the low glucose release observed for crude enzymes from WB/L and GPW/MW was due to a difference in the nitrogen source. The MW, which was used as an alternative nitrogen supplement was not efficient for GPW as it only resulted in glucose release of 2.70 g/L. This may be because the MW contains inhibitory compounds that negatively affect the growth of T. reesei. The nitrogen source added to the GPW/N medium was urea and $(NH_4)_2SO_4$ and may have been the main factor for improved enzyme production of both *A. carbonarius* and *T. reesei* since it has been shown that $(NH_4)_2SO_4$ was one of the optimal nitrogen sources for enzyme production in *A. niger* [23]. Therefore, it was analyzed whether *T. reesei* grown on the same GPW substrate without supplemented nitrogen could yield as high enzyme production related to wheat straw hydrolysis. As can be seen in Fig. 5.8 there was a clear reduction in the potential of the crude enzymes after growth in a nitrogen reduced medium, resulting in glucose release levels of only 1.8 g/L. Furthermore, by supplementing WB/L with the same nitrogen sources that were used in GPW/N medium, the glucose release increased from 1.9 to 2.8 g/L.

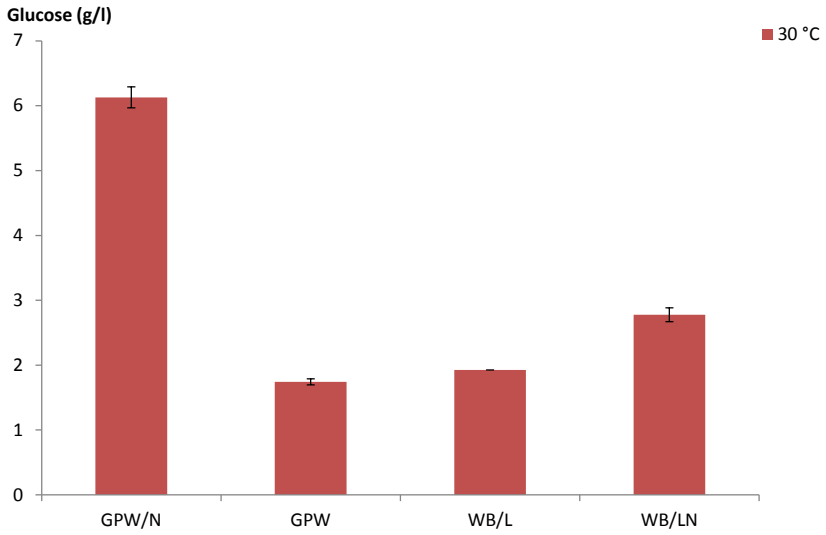


**Figure 5.8** Glucose release after 5 % wheat straw hydrolysis using crude enzymes obtained from *T. reesei* Rut-C30 cultivations on GPW/N, GPW, WB/L, WB/LN medium at 30 °C. Error bars denote SD (N = 2).

## 5.5 Conclusion

The optimal medium for cellulase production by *T. reesei* Rut-C30 was found to be GPW/N medium, which resulted in the highest glucose release from wheat straw hydrolysis of 6.1 g/L, and highest avicelase, xylanase and β-glucosidase compared to WB/L, GPW/MW and WB/SP media. The effect of the nitrogen source added to the GPW/N medium was found to be highly significant for improved activity in wheat straw hydrolysis, due to a drastic decrease to 1.8 g/L in glucose release when nitrogen was removed from the medium. For GPW to be applied as a cultivation substrate for on-site enzyme production in a commercial setting the supplementation of additional nitrogen sources urea and $(NH_4)_2SO_4$ are required. For *A. carbonarius* the medium that elicited optimal wheat straw hydrolysis was also GPW/N, however, for avicelase, xylanase and β-glucosidase the optimal medium was WB/L and WB/SP. Furthermore, it was found that an optimal cultivation time for β-glucosidase production by *A. carbonarius* was 10 days in WB/SP medium, and that the optimal temperature was 30 °C. This temperature was found to be optimal for cultivation in all media analyzed as observed by generally higher activities. Even though there was highest β-glucosidase activity after 10 days in WB/SP, the same crude enzymes had a lower avicelase activity. For an on-site enzyme production using *A. carbonarius* the highest activities of xylanase, avicelase and β-glucosidase would be achieved by using WB/SP medium and cultivation at 30 °C. However, the duration of the cultivation may not be optimal at 10 days due to the observed lower avicelase. Relative to the wheat straw hydrolysis efficiency the GPW/N medium appears as an attractive choice for on-site enzyme production using both *A. carbonarius* and *T. reesei* Rut-C30 if the cultivation time is 5 days. The decreasing activities in GPW/N medium at prolonged cultivation times may be alleviated by supplementation of water

during the cultivation. This medium would be a cheap alternative to conventional cellulase production and could be applied in a consolidated bioprocess setup. For a consolidated process using GPW/N a co-cultivation of *T. reesei* Rut-C30 and *A. carbonarius* may elicit efficient utilization of the substrate and an improved production of enzymes organic acids. This may also be implemented using only *A. carbonarius* as there was a minimal difference in the wheat straw hydrolysis efficiency compared to *T. reesei* Rut-C30.

## 5.6 Acknowledgements

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## 5.7 References


[1] Frisvad JC, Larsen TO, Thrane U, Meijer M, Varga J, Samson RA, et al. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS One 2011;6:e23496.

[2] Guais O, Borderies G, Pichereaux C, Maestracci M, Neugnot V, Rossignol M, et al. Proteomics analysis of "Rovabiot Excel", a secreted protein cocktail from the filamentous fungus *Penicillium funiculosum* grown under industrial process fermentation. J Ind Microbiol Biotechnol 2008;35:1659–68.

[3] Haltrich D, Nidetzky B, Kulbe KD, Zupancic S. Production of fungal xylanases. Bioressource Technol 1997;58:137–61.

[4] Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, et al. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 2007;25:221–31.

[5] Ward OP. Production of recombinant proteins by filamentous fungi. Biotechnol Adv 2012;30:1119–39.

[6] Bubner P, Plank H, Nidetzky B. Visualizing cellulase activity. Biotechnol Bioeng 2013;110:1529–49.

[7] Levasseur A, Drula E, Lombard V, Coutinho PM, Henrissat B. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. Biotechnol Biofuels 2013;6:41.

[8] Rodriguez-Zúñiga UF, Cannella D, Giordano RDC, Giordano RDLC, Jørgensen H, Felby C. Lignocellulose pretreatment technologies affect the level of enzymatic cellulose oxidation by LPMO. Green Chem 2015;17:2896–903.

[9] Rana V, Eckard AD, Teller P, Ahring BK. On-site enzymes produced from *Trichoderma reesei* RUT-C30 and *Aspergillus saccharolyticus* for hydrolysis of wet exploded corn stover and loblolly pine. Bioresour Technol 2014;154:282–9.

[10] Prévot V, Lopez M, Copinet E, Duchiron F. Comparative performance of commercial and laboratory enzymatic complexes from submerged or solid-state fermentation in lignocellulosic biomass hydrolysis. Bioresour Technol 2013;129:690–3.

[11] Hansen GH, Lübeck M, Frisvad JC, Lübeck PS, Andersen B. Production of cellulolytic enzymes from ascomycetes: Comparison of solid state and submerged fermentation. Process Biochem 2015;50:1327–41.

[12] Nasir Z, Uellendahl H. Enhancing the hydrolysis process of a two-stage biogas technology for the organic fraction of municipal solid waste. Biogas Sci 2014 n.d.

[13] Kolasa M, Ahring BK, Lübeck PS, Lübeck M. Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. Bioresour Technol 2014;169:143–8.

[14] Lang C. Simple microdetermination of Kjeldahl nitrogen in biological materials. Anal Chem 1958;30:1692–4.

[15] Mandels M, Weber J. The production of cellulases. Adv Chem Ser 1969;95:394–414.

[16] Sørensen A, Lübeck PS, Lübeck M, Teller PJ, Ahring BK. β -Glucosidases from a new *Aspergillus* species can substitute commercial β -glucosidases for saccharification of lignocellulosic biomass. Can J Microbiol 2011;650:638–50.

[17] Herpoël-Gimbert I, Margeot A, Dolla A, Jan G, Mollé D, Lignon S, et al. Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. Biotechnol Biofuels 2008;1:18.

[18] Leong S-LL, Hocking AD, Scott ES. Effect of temperature and water activity on growth and ochratoxin A production by Australian *Aspergillus carbonarius* and *A. niger* isolates on a simulated grape juice medium. Int J Food Microbiol 2006;110:209–16.

[19] Kiss T, Kiss L. Purification and characterization of an extracellular beta-D-xylosidase from *Aspergillus carbonarius*. World J Microbiol Biotechnol 2000;16:465–70.

[20] Meijer M, Houbraken JAMP, Dalhuijsen S, Samson RA, de Vries RP. Growth and hydrolase profiles can be used as characteristics to distinguish *Aspergillus niger* and other black aspergilli. Stud Mycol 2011;69:19–30.

[21] Stricker AR, Steiger MG, Mach RL. Xyr1 receives the lactose induction signal and regulates lactose metabolism in Hypocrea jecorina. FEBS Lett 2007;581:3915–20.

[22] Warzywoda M, Ferre V, Pourquie J. Development of a culture medium for large-scale production of cellulolytic enzymes by *Trichoderma reesei*. Biotechnol Bioeng 1983;25:3005–11.

[23] Hanif A, Yasmeen A, Rajoka MI. Induction, production, repression, and de-repression of exoglucanase synthesis in *Aspergillus niger*. Bioresour Technol 2004;94:311–9.

[24] Rocky-Salimi K, Hamidi-Esfahani Z. Evaluation of the effect of particle size, aeration rate and harvest time on the production of cellulase by *Trichoderma reesei* QM9414 using response surface methodology. Food Bioprod Process 2010;88:61–6.

[25] Kabel MA, van der Maarel MJEC, Klip G, Voragen AGJ, Schols HA. Standard assays do not predict the efficiency of commercial cellulase preparations towards plant materials. Biotechnol Bioeng 2006;93:56–63.

# Chapter 6

# Fungal enzyme production and biomass determination in solid state fermentation

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# Fungal enzyme production and biomass determination in solid state fermentation

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## 6.1 Abstract

Two indirect methods for quantification of fungal biomass were evaluated, based on measurements of the cell specific components ergosterol (Erg) and β-N-acetylhexosaminidase (NAHA). The methods were tested on four fungi, *Aspergillus carbonarius*, *A. saccharolyticus*, *Talaromyces pinophilus* and *Trichoderma reesei* for evaluation of their growth in a solid state medium. It was observed that measurements of Erg yielded more reproducible data with a low standard deviation of 2 – 8 %, compared to the less reproducible NAHA values with a standard deviation of 11 – 23 %. Erg measurements also generated more reliable values as it correlated well with expected increases in biomass. For all fungi the Erg value increased when cultivation was prolonged to 14 days, relative to the measurements at 7 days. The endoglucanase and β-glucosidase activity also increased with prolonged cultivation time. Enzyme activities increased less than the corresponding Erg values, indicating that enzyme activity is not linearly correlated to biomass.

Keywords: Solid state fermentation, Fungal biomass quantification, Ergosterol, β-*N*-acetylhexosaminidase, Enzyme production.

## 6.2 Introduction

Determination of fungal biomass is necessary in both industrial applications of filamentous fungi and for determination of growth kinetics in order to assess optimal parameters for growth and production of desired compounds such as enzymes [1,2]. Industrial scale solid state fermentation (SSF) is being used by several companies to produce enzymes [3–5], however, the determination of fungal biomass in such systems is complicated because fungal mycelium and solid medium cannot be separated. Thus estimation of fungal biomass must be solved indirectly by quantification of chemical markers, such as the cell membrane constituents ergosterol (Erg), chitin (as its monomer glucosamine), fatty acids or nucleic acids [6–11]. Also measurements of biological activity in the form of ATP, enzyme activity of β-N-acetylhexosaminidase (NAHA), respiration rate ($CO_2$), protein and immunological activity have been used for estimation of fungal biomass in SSF [12–16]. All of the methods have their pros and cons, and in a recent study eleven of the methods including Erg, glucosamine, protein, genomic DNA, respiration rate and enzyme activity were evaluated and compared in relation to SSF of *Trametes hirsuta* [17]. They found that dry biomass had a high linear correlation with Erg and glucosamine content, and that Erg measurements were the most reliable as it followed the expected growth curves and had a high reproducibility [17]. Based on this and the fact that Erg is the most abundant and resilient compound in cell membranes of fungi and yeasts, it is the preferred choice for determination of biomass [18].

While analysis of Erg content is well correlated to biomass [19] the extraction of Erg is still a laborious and time consuming process [17]. Therefore, it would be advantageous to have an alternative and faster method for determining fungal growth in SSF. This could be achieved by measuring the activity of the enzyme β-N-acetylhexosaminidase (NAHA), which is present in both spores and mycelium (living and dead). The NAHA activity is measured using a substrate that releases a fluorophore (4-methylumbelliferone) upon cleavage, and it has been shown to correlate with the fungal growth markers Erg and phospholipid fatty acid 18:2ω6 in soil samples [13]. The method has been commercialized as MycoMeter-Test™ and used for detection and quantification of fungal growth on surfaces in mouldy buildings [20]. This method is also linearly correlated to Erg content on agar media [21] and building materials [22].

The objective of this work was to develop and test methods for determining and quantifying the biomass of several cellulase producing industrially relevant fungi in SSF by use of Erg and NAHA. These were evaluated by assessing fungal biomass for *Aspergillus carbonarius*, *A. saccharolyticus*, *Talaromyces pinophilus*, and *Trichoderma reesei* Rut-C30 and furthermore measuring enzyme production relative to the biomass.

## 6.3 Methods and materials

### 6.3.1 *Fungal strains*

The strains used were: *A. carbonarius* (ITEM 5010), *A. saccharolyticus* (IBT 28231), *Talaromyces pinophilus* (IBT 29404) and *Trichoderma reesei* Rut-C30 (ATCC 56765). The strains were maintained in 10 % glycerol at -80 °C. Laboratory cultures were maintained on V8 juice agar (V8) plates [23] for *A. carbonarius*, *A. saccharolyticus* and *Tr. reesei*. Malt extract agar (MEA) plates [23] were used to maintain *Ta. pinophilus*.

### 6.3.2 *Solid state fermentation*

The medium used for solid state fermentation, consisted of wheat bran (WB) mixed with sphagnum peat (WB/SP), in accordance to the procedure described by Kolasa et al. [24]. For each fungal strain three 250 mL Erlenmeyer shake flasks with WB/SP medium were prepared and each flask was inoculated using three agar plugs. The plugs were taken with a plug borer (1 cm diameter) from actively growing cultures on V8 plates, in the case of *Ta. pinophilus* from MEA plates. The fungi were cultivated for 7 and 14 days at 25 °C and the flasks were shaken twice a day by hand for aeration and equal distribution of the spores.

### 6.3.3 *Enzyme extraction*

After cultivation the enzymes were extracted by addition of 50 mL Milli-Q water, and the flasks were left O/N at 4 °C with 180 rpm shaking. The liquid supernatant was separated by pelleting the solid substrate and fungal biomass at 10,000 x G for 20 min and hereafter decanting the supernatant into new 50 mL falcon tubes. The supernatant was stored at -20 °C prior to use in enzyme assays. The pellet was freeze-dried for 48 hours, to obtain a dry sample of mixed substrate and fungal biomass (MSB) applicable for Erg and NAHA measurements.

### 6.3.4 *Ergosterol extraction and measurement*

The freeze-dried MSB pellets obtained from the enzyme separation were mixed rigorously by hand stirring and grinding with a metal spatula to ensure homogeneity. Three hundred milligrams of the substrate/fungal biomass mixture was transferred to 15 mL falcon tubes. One milliliter of NaOH (2 M) and 4 mL of methanol were added to the sample tubes, which were then vortexed and kept at 85 °C for 90 min with vortexing every 30 min. When the samples had cooled to room temperature, 6 mL of pentane was added and the mixture was vortexed. After phase separation 4.5 mL from the upper pentane phase was transferred to a 16 mL glass vial for evaporation under $N_2$ at 42 °C. Dry samples were re-dissolved in 1 mL of

isopropanol with 250 rpm shaking at room temperature for 30 min. Re-dissolved samples were filtered through 0.45 µm PTFE filters into HPLC vials, and stored at -20 °C prior to HPLC analysis.
HPLC was performed by reverse phase chromatography on a Dionex Ultimate 3000 (Sunnyvale, CA, USA) UHPLC, equipped with a Dionex 3000 RS Diode Array Detector and a Poroshell 120 Phenyl-Hexyl column (Agilent, Santa Clara, CA, USA). An injection volume of 7.5 µL was used in conjunction with a flow rate of 0.6 mL/min at 40 °C. Eluent A was 50 ppm trifluoroacetic acid in Milli-Q water, and eluent B was 50 ppm TFA in acetonitrile. For elution a gradient of 6.5 min was used, starting at 50 % solvent B with a linear gradient to 80 % B over 1 min, then linearly increased to 100 % B over 1 min and maintained at this level for 2 min, followed by a decreasing gradient to 50 % B for 0.5 min and maintained at this level for 2 min.

Quantification was achieved using a blank matrix spiked at 7 levels and based on the peak area at 282 nm (±2 nm) chromatogram, and the identity of Erg was verified by the retention time ± 0.01 and the distinctive UV-spectrum. Freeze-dried matrix, 300 mg, was spiked with 50 µL to final concentrations of 1, 0.75, 0.50, 0.25, 0.10, 0.05 and 0 mg Erg/mL and left 2h to dry. These spiked samples were extracted following the same procedure as described above. Recovery of Erg was determined by comparing samples spiked with 0.75, 0.25 and 0.05 mg Erg/mL before and after extraction.

### 6.3.5 β-*N-acetylhexosaminidase activity measurement*

The NAHA activity was measured following the MycoMeter protocol [21] with minor modifications. Briefly, 1.5 mL enzyme substrate was added to 100 mg of homogenized MSB and mixed thoroughly. Incubation was performed in a Thermomixer comfort (Eppendorf) at 23 °C, for 30 min with 600 rpm shaking. Thereafter solids were quickly spun down in a table centrifuge at 15 000 g and 100 µL of the reaction volume was transferred to a cuvette containing 2 mL developer solution and mixed. NAHA activity was measured with a fluorometer (MycoMeter Aps) and expressed as arbitrary fluorescence units denoted as NAHA values.

### 6.3.6 *AZCL enzyme assay*

The supernatants obtained from triplicate growth of each fungus were analyzed for activities using 5 different Azurine cross-linked (AZCL) substrates: arabinan, arabinoxylan (birchwood), cellulose, galactan and galactomannan (Megazyme, Bray, Ireland). To prepare the different AZCL assays, a stock solution with phosphoric acid (0.08 M) (Merck, Ortho-Phosphoric acid 85 %), glacial acetic acid (0.08 M) (Merck, 100%) and boric acid (0.08 M) (Merck) was made using double distilled water. To prepare the different AZCL-assay, 200 mL double distilled water was added to 144 mL stock solution. The pH was adjusted to 6 and double distilled water was added again to give a total volume of 500 mL. Agarose, 7.5 g (Litex, HSB 200

Protein grade) was added and the solution was heated in a microwave oven until the agarose had dissolved. For each AZCL assay, 500 mg of azurine bound substrate was presoaked in 96 % ethanol for 10 min before use. When the agarose solution had cooled to approximately 65 °C, the AZCL substrate was added while stirring. The suspension was poured into Petri dishes with a 9 cm diameter and 8 wells with a 4 mm diameter were cut in the solidified agar plates. For each enzyme extract 35 µL was added to a well and a filled plate was incubated for 24 h at 30 °C. The activity of each enzyme was measured as the radius of the zone of released azurine dye (the blue halo) around each application well.

### 6.3.7 β-*glucosidase assay*

β-glucosidase activity was measured using 5 mM para-nitrophenyl-β-D-glucopyranoside (pNPG) (Sigma Aldrich) in 50 mM sodium citrate (pH 4.8) as the substrate. The assay was carried out in a microtiter-plate format according to [25]. A 10 µL volume of sample supernatant was added to 100 µL substrate in 1.5 mL Eppendorf tubes, and incubated in a Thermomixer® comfort (Eppendorf) at 50 °C for 15 min. At the end of the reaction 60 µL of the reaction volume was transferred to a Greiner 96 well microtiter plate (Sigma Aldrich) already containing 100 µL 1 M $Na_2CO_3$ for termination of the reaction. Absorbance at 405 nm was measured in a plate reader (BioTek, EL800). Para-nitrophenol was used for preparation of a standard curve, and one unit (U) of enzyme activity was defined as the amount of hydrolyzed pNPG (µmol) in 1 min per volume of enzyme extract (mL).

## 6.4 Results and discussion

### 6.4.1 *Quantification of Erg and NAHA*

For the determination of ergosterol (Erg) a standard curve was made from a blank matrix (non-inoculated WB/SP medium, freeze dried) spiked with seven different concentrations of Erg. The resulting calibration curve showed a linear correlation ($R^2$ = 0.9916) between the concentration and the area under the curve (AUC) observed at 282 nm (Appendix 1). The amount of Erg was calculated based on the standard curve (Appendix 1) and reported relative to one gram of homogenized freeze dried substrate and biomass (MSB) spiked with Erg.

The recovery of Erg was evaluated at 0.05, 0.25 and 0.75 mg/mL from samples prepared by spiking the blank matrix before and after extraction. Similar recoveries (30 – 40 % mg/mL ± 2-13 %) were obtained for the Erg at the different concentrations tested (Appendix, Fig. A2). These results show that a standard curve made from blank matrix samples spiked before extraction is required to account for the loss in the

extraction, rather than an external standard curve of pure Erg standards. For assessing the loss of Erg during the liquid-liquid extraction, re-extraction of the samples was done by adding a second round of pentane. The amount of Erg determined in the second round of pentane extraction corresponded to 2 – 20 % of the total amount from the first and second extraction (data not shown).

Stability of extracted Erg has been proven by Gessner et al. [26] where no loss was observed for samples stored in methanol at room temperature for 1 week, and at -20 °C the samples could be stored 4-6 months with losses of 4 – 21 %. In a study by Mille-Lindblom et al. [27] sterilized soil samples only had ergosterol losses of 34 % after 2 months storage, however, treatment with sunlight rapidly decreased the ergosterol amount due to the UV transforming it into ergocalciferol.

Biomass measurements of the four fungi analyzed in this study have to the extent of our knowledge, not been investigated in SSF before. At time zero the amount of Erg present in the medium was only 5 µg pr. g wheat bran sphagnum peat medium. It was clear that *Talaromyces pinophilus* produced highest amount of Erg with 3.56 ± 0.27 mg/g MSB after 7 days cultivation and 5.70 ± 0.38 mg/g MSB after 14 days cultivation (Fig. 6.1, g), indicating a higher growth rate compared to the Aspergilli and *Trichoderma reesei*. Erg measurements of hyphae have shown increased values with time and mycelium age while Erg remained present in dead hyphae, therefore the method overestimates live mycelium [13]. It may be assumed that the same overestimation applies to all of the fungi thereby enabling that Erg can still be used for biomass and growth comparison. The large increase in Erg for *Ta. pinophilus* from 7 days to 14 days, could indicate a continued high growth even after 7 days, compared to *A. carbonarius* where the Erg only increased by 0.33 mg/g MSB (20.5 %). The *A. carbonarius* did therefore not seem to grow much more after the 7 days cultivation. This also correlates to a small increase in the enzyme activities of *A. carbonarius*, whereas *Ta. pinophilus* showed a larger enzyme activity increase at prolonged cultivation, which matched the high Erg accumulation. For both *Tr. reesei* and *A. saccharolyticus* there was a significant increase in the amount of Erg, 0.85 and 1.01 mg/g MSB respectively, after 14 days cultivation showing that both fungi continued to grow after 7 days in contrast to *A. carbonarius*.

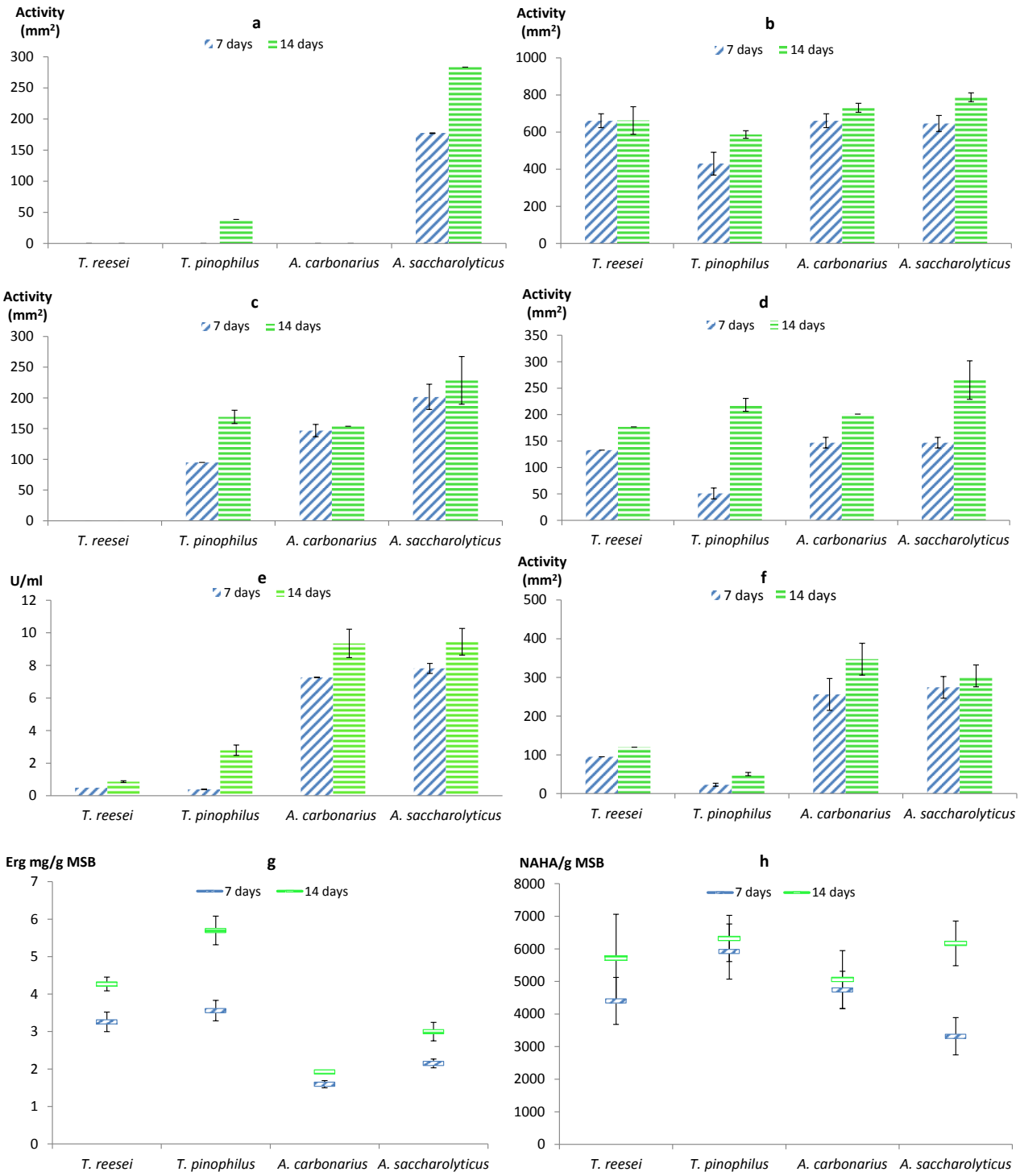


**Figure 6.1** Enzyme activity for a) arabinanase, b) endo-β-1,4-xylanase, c) galactanase d) endoglucanase, e) β-glucosidase, f) β-mannanase, g) Erg (mg/g mixed substrate biomass (MSB)), h) NAHA activity/g MSB after 7 and 14 days, for *Ta. pinophilus*, *Tr. reesei*, *A. carbonarius* and *A. saccharolyticus*. Error bars denote standard deviation (n = 3 for a - f and n = 6 for g - h)

Furthermore it appears that all four fungi reached stationary growth phase before 14 days cultivation as the increase in Erg was not doubled or exponentially increased during the extra cultivation time. This may

be an effect of the water activity decreasing and the medium drying out. The increase in Erg for *Ta. pinophilus* correlates well with the increased enzyme activities of endoglucanase, β-glucosidase, galactanase and arabinanase (Fig. 6.1, a and c-e). The other enzyme activities mannanase and endo-xylanase reached a plateau and did not increase with the continued growth. Jørgensen et al. [28] reported a similar trend for *Ta. pinophilus* of increasing endoglucanase, β-glucosidase and galactanase activity with longer cultivation time while mannanase and endo-xylanase activities increased only until 5 – 6.5 days. For all fungi it was observed that the prolonged growth led to increased endoglucanase (Fig. 6.1, d). The increased enzyme activities are not linearly proportional to the increased Erg values showing that a high biomass does not equal a high enzyme production or activity. This is corroborated by the difference in amount of biomass (Erg) between *A. carbonarius* and *Ta. pinophilus*, which had similar activities of endo-glucanase, galactanase and endo-xylanase after 14 days (Fig. 6.1: b, c and d).

NAHA provides a much faster method for determination of fungal biomass, as it can be done in only 1 hour compared to days of labor for the Erg extraction. However, the standard deviations of the NAHA values obtained in this study ranged from 11 to 23 % as seen in Fig. 6.1 (h). In a study by Klamer et al. [29] they used NAHA values to quantify fungal biomass of *Aspergillus versicolor*, *Penicillium chrysogenum* and *Trichoderma viride* on surfaces of building materials and also obtained high standard deviations > 20 %. Also the trend of increased growth observed from the Erg measurements was not as apparent when evaluating the NAHA values. Although, the same trend of *Ta. pinophilus* having the highest values after 7 days cultivation (Fig. 6.1, h) does concur with Erg measurements, there is still a discrepancy for *A. carbonarius* which appeared to have the second most biomass based on NAHA values. Only *A. saccharolyticus* showed an increased biomass from 7 to 14 days, the three others had similar levels of NAHA values or biomass at both days. The unchanging NAHA values in this study might be caused by the stability of the NAHA enzyme in a medium that did not receive additional water during 14 days, as this might have led to drying out and thereby also autolysis. This would have a negative impact on the NAHA enzyme due to the release of intracellular proteases, which might degrade NAHA. In the study by Reeslev et al. [21] NAHA enzyme activity and ergosterol yielded very similar information regarding fungal biomass over time, for *A. versicolor* and *Stachybotrys chartarum*. The data suggested that interspecies differences may affect the enzyme activity more than the ergosterol amount [21]. The discrepancy between the results of Reeslev et al. [21] and those obtained in this study may be due to the difference in fungal strains or the medium applied, which may affect the measurements.

The NAHA values, which indicate that only *A. saccharolyticus* increased in biomass, did not concur with the increasing enzyme activities observed for all four fungi. Although the NAHA method showed a higher

standard deviation of 11 - 23 % compared to the 2 – 8 % observed for the Erg measurements, it still serves as a fast indicator of growth in SSF. Furthermore, there is a higher possibility of turning the NAHA method into an automated and therefore high throughput screening rather than the Erg method, which cannot be automated and requires expensive equipment. Also the standard deviation of the NAHA values could be lowered by further optimization of the method e.g. by applying a larger amount of MSB sample or measuring on samples that have not been through an enzyme extraction and freeze drying.

Due to the high standard deviations observed for NAHA values and the lack of correlation between these values and the increased enzyme activities obtained after 14 days cultivation, it would at the present time not be an ideal method for quantification of fungal biomass in SSF. Therefore for future biomass determinations in SSF it would be more reliable to use measurements of Erg, as the increasing values after 14 days cultivation are more trustworthy when correlated to the increased enzyme activities. However, the Erg method presented here is still a time consuming process

### 6.4.2 *AZCL and β-glucosidase enzyme activities*

The AZCL screening showed that all four fungi produced relatively high amounts of endo-glucanase from 50 to 147 $mm^2$ after 7 days (Fig. 6.1, d), as expected from previous reports on cellulolytic activity of these fungi [24,30]. All four fungal strains also expressed high endo-xylanase activity ranging from 429 to 646 $mm^2$ after 7 days growth on WB/SP medium. This effect may be related to the high amount of glucuronoarabinoxylan (28 %) present in WB [31]. The β-mannanase activity was significantly higher for both *A. carbonarius* (347 $mm^2$) and *A. saccharolyticus* (303 $mm^2$) whereas *Tr. reesei* and *Ta. pinophilus* produced the lowest activities of 119 and 50 $mm^2$, respectively. Galactanase activity was highest for both Aspergilli after 7 days cultivation, but *Ta. pinophilus* had a higher activity than *A. carbonarius* after 14 days. It was also apparent that neither *A. carbonarius* nor *Tr. reesei* expressed any arabinanase activity and that interestingly *Ta. pinophilus* only expressed it after 14 days cultivation. The broadest enzyme profile was observed for *A. saccharolyticus*, since it produced all the 6 analyzed activities. A slight increase was observed for all analyzed activities except the endo-xylanase for *Tr. reesei* and galactanase for *A. carbonarius* crude enzymes extracted from 14 days cultivations. These increases may be an effect of increased protein/enzyme concentration, due to a continuous expression. Especially endoglucanase activity improved with 167 and 118 $mm^2$ larger activity area for *Ta. pinophilus* and *A. saccharolyticus* and around 50 $mm^2$ more for *Tr. reesei* and *A. carbonarius* after 14 days cultivation. This shows that higher cellulase activities may be achieved by prolonging the cultivation from 7 to 14 days, possibly due to increased protein/enzyme concentration.

Both *A. carbonarius* and *A. saccharolyticus* also produced significantly higher activities of β-glucosidase on day 14 (9.4 and 9.5 U/mL) relative to day 7 (7.3 and 7.8 U/mL). for *Tr. reesei* and *Ta. pinophilus* the activities also increased from 0.4 to 2.8 U/mL. The high activity of the two Aspergilli were as expected although it was less than previous reports of 20 U/mL for crude enzymes of both fungi and 339 U/mL for 10 x concentrated enzymes of *A. saccharolyticus* [24,32]. The low activity observed for *Ta. pinophilus* and *Tr. reesei* were expected, due to previous reports of similar results [33,34]. Prolonged cultivation resulted in increased β-glucosidase activity for all fungi except *Tr. reesei*. However, the increases in all enzyme activities were in general not linearly correlated to the Erg (biomass) measurements. This shows that it is advantageous to apply measurements of the desired enzyme activities during growth characterization as an increased biomass does not correlate to a high enzyme activity.

## 6.5 Conclusion

For quantification of *A. carbonarius*, *A. saccharolyticus*, *Ta. pinophilus* and *Tr. reesei* biomass in a solid state medium containing wheat bran and sphagnum peat, the optimal method was measurements of Erg. This method, although it takes days to perform, gave the most reproducible and trustworthy results. However, the extraction protocol applied only yielded a recovery ranging from 30 – 40 %. The amount of Erg of the four fungi increased when the cultivation period was prolonged to 14 days, indicating increased biomass and therefore continued growth. The same trend was not apparent when applying the NAHA value as a measurement of biomass, and the NAHA values were also affected by high standard deviations of 11 - 23 %. Despite the fact that NAHA values could be obtained much faster, it would not be optimal to apply it for tracking fungal biomass in solid state fermentations. The enzyme activities measured for *Ta. pinophilus* correlate well with the increasing biomass (Erg based), as the same tendencies were observed in a submerged fermentation setup [28]. For all four fungi there was an increase in the measured endoglucanase activity after 14 days cultivation, and all fungi also had an increase in β-glucosidase activity. Enzyme activity increases were though not linearly correlated to the increases in measured Erg (biomass). The approach of acquiring both enzymes and sample for biomass determination could be applied in future studies of optimal enzyme production in SSF.

## 6.6 Acknowledgement

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## 6.7 References


[1] Krishna C. Solid-state fermentation systems-an overview. Crit Rev Biotechnol 2005;25:1–30.

[2] Bhargav S, Panda B, Ali M, Javed S. Solid-state fermentation systems-an overview. Crit Rev Biotechnol 2008;25:1–30.

[3] Durand A, Maratray J, Almanza S, Pelletier A, Renaud R. Reactor for sterile solid-state fermentation methods. Patent. EP 0683815 B1, 1996.

[4] Hooge DM, Pierce JL, McBride KW, Rigolin PJ. Meta-analysis of broiler chicken trials using diets with or without allzyme SSF enzyme complex. Int J Poult Sci 2010;9:819–23.

[5] Suryanarayan S, Mazumdar K. Solid state fermentation. Patent. US 6664095 B1, 2003.

[6] May B, VanderGheynst J, Rumsey T. The kinetics of *Lagenidium giganteum* growth in liquid and solid cultures. J Appl Microbiol 2006;101:807–14.

[7] Young J. Microwave-assisted extraction of the fungal metabolite ergosterol and total fatty acids. J Agric Food Chem 1995;43:2904–10.

[8] Szponar B, Larsson L. Determination of microbial colonisation in water-damaged buildings using chemical marker analysis by gas chromatography-mass spectrometry. Indoor Air 2000;10:13–8.

[9] Miller J, Young J. The use of ergosterol to measure exposure to fungal propagules in indoor air. Am Ind Hyg Assoc J 1997;58:39–43.

[10] Lin H, Cousin M. Detection of mold in processed foods by high performance liquid chromatography. J Food Prot 1985;48:671–8.

[11] Pasanen A, Yli-Pietilä K, Pasanen P, Kalliokoski P, Tarhanen J. Ergosterol content in various fungal species and biocontaminated building materials. Appl Environ Microbiol 1999;65:138–42.

[12] Larroche C, Gros J. Characterization of the growth and sporulation behavior of *Penicillium roquefortii* in solid substrate fermentation by material and bioenergetic balances. Biotechnol Bioeng 1992;39:815–27.

[13] Miller M, Palojärvi A, Rangger A, Reeslev M, Kjøller A. The use of fluorogenic substrates to measure fungal presence and activity in soil. Appl Environ Microbiol 1998;64:613–7.

[14] Dubey A, Suresh C, Kumar S, Karanth N. An enzyme-linked immunosorbent assay for the estimation of fungal biomass during solid-state fermentation. Appl Microbiol Biotechnol 1998;50:299–302.

[15] Ikasari L, Mitchell DA. Oxygen uptake kinetics during solid state fermentation with *Rhizopus oligosporus* 1998;12:171–5.

[16] Gutierrez-Correa M, Tengerdy RR. Xylanase production by fungal mixed culture solid substrate fermentation on sugar cane bagasse. Biotechnol Lett 1998;20:45–7.

[17] Steudler S, Bley T. Biomass estimation during macro-scale solid-state fermentation of basidiomycetes using established and novel approaches. Bioprocess Biosyst Eng 2015;38:1313–23.

[18] Mercer EI, Bartlett K. Sterol esters of Phycomyces blakesleeanus. Phytochemistry 1974;13:1099–105.

[19] Matcham S, Jordan B, Wood D. Estimation of fungal biomass in a solid substrate by three independent methods. Appl Microbiol Biotechnol 1985;21-21:108–12.

[20] Reeslev M, Miller M. The MycoMeter(TM)-Test: A new rapid method for detection and quantification of mold in buildings. Proc Heal Build 2000;1:589–90.

[21] Reeslev M, Miller M, Nielsen KF. Quantifying mold biomass on gypsum board: Comparison of ergosterol and beta-N-acetylhexosaminidase as mold biomass parameters. Appl Environ Microbiol 2003;69:5–8.

[22] Nielsen KF, Holm G, Uttrup LP, Nielsen PA. Mould growth on building materials under low water activities. Influence of humidity and temperature on fungal growth and secondary metabolism. Int Biodeterior Biodegrad 2004;54:325–36.

[23] Samson R, Houbraken J, Thrane U, Frisvad J, Andersen B. Food and Indoor Fungi. 1st ed. CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands; 2010.

[24] Kolasa M, Ahring BK, Lübeck PS, Lübeck M. Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. Bioresour Technol 2014;169:143–8.

[25] Sørensen A, Lübeck PS, Lübeck M, Teller PJ, Ahring BK. β -Glucosidases from a new *Aspergillus* species can substitute commercial β -glucosidases for saccharification of lignocellulosic biomass. Can J Microbiol 2011;650:638–50.

[26] Gessner MO, Bauchrowitz MA, Escautier M. Extraction and quantification of ergosterol as a measure of fungal biomass in leaf litter. Microb Ecol 1991;22:285–91.

[27] Mille-Lindblom C, Von Wachenfeldt E, Tranvik LJ. Ergosterol as a measure of living fungal biomass: Persistence in environmental samples after fungal death. J Microbiol Methods 2004;59:253–62.

[28] Jørgensen H, Mørkeberg A, Krogh KBR, Olsson L. Production of cellulases and hemicellulases by three *Penicillium* species: effect of substrate and evaluation of cellulase adsorption by capillary electrophoresis. Enzyme Microb Technol 2005;36:42–8.

[29] Klamer M, Morsing E, Husemoen T. Fungal growth on different insulation materials exposed to different moisture regimes. Int Biodeterior Biodegradation 2004;54:277–82.

[30] Krogh KBR, Mørkeberg A, Jørgensen H, Frisvad JC, Olsson L. Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes. Appl Biochem Biotechnol 2004;113-116:389–401.

[31] Maes C, Delcour J. Alkaline hydrogen peroxide extraction of wheat bran non-starch polysaccharides. J Cereal Sci 2001;34:29–35.

[32] Rana V, Eckard AD, Teller P, Ahring BK. On-site enzymes produced from *Trichoderma reesei* RUT-C30 and *Aspergillus saccharolyticus* for hydrolysis of wet exploded corn stover and loblolly pine. Bioresour Technol 2014;154:282–9.

[33] Visser EM, Falkoski DL, de Almeida MN, Maitan-Alfenas GP, Guimarães VM. Production and application of an enzyme blend from *Chrysoporthe cubensis* and *Penicillium pinophilum* with potential for hydrolysis of sugarcane bagasse. Bioresour Technol 2013;144:587–94.

[34] Herpoël-Gimbert I, Margeot A, Dolla A, Jan G, Mollé D, Lignon S, et al. Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. Biotechnol Biofuels 2008;1:18.

# Chapter 7

# LPMO increases glucose and xylose yield in wheat straw hydrolysis when supplemented to crude enzymes of *Aspergillus carbonarius*

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This chapter presents the results achieved by supplementation of purified LPMO to the crude enzymes of *A. carbonarius* for investigation of synergy in hydrolysis of pretreated wheat straw.

# LPMO increases glucose and xylose yield in wheat straw hydrolysis when supplemented to crude enzymes of *Aspergillus carbonarius*

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## 7.1 Abstract

Application of *Aspergillus carbonarius* crude enzymes for hydrolysis of wheat straw had a cellulose conversion efficiency of 23 %. In this study it was shown that supplementation with purified lytic polysaccharide monooxygenase (LPMO) from *Thermoascus aurantiacus* (Ta_LPMO) boosts the crude enzymes and increases efficiency by 6.8 %. The efficiency could be further improved to 8.5 % by addition of 10 mM ascorbate, showing that the effectiveness of an LPMO is dependant on the type and concentration of the electron donor. An LPMO of *Podospora anserina* (Pa_LPMO) and a cellobiose dehydrogenase (CDH) from *Neurospora crassa* (Nc_CDH) was expressed in *A. carbonarius.* The resulting crude enzymes of the Pa_LPMO, Nc_CDH mutants showed an improved xylan conversion, by 1.2 g/L increased xylose release from wheat straw hydrolysis. It can therefore be concluded that LPMO's are efficient boosters of *A. carbonarius* crude enzymes and can be used to significantly improve lignocellulose hydrolysis, but the results depend on origin and type of the LPMO.

Keywords: AA9, crude enzyme hydrolysis, *Aspergillus carbonarius*, electron donors, *Podospora anserina*, *Neurospora crassa*, *Thermoascus aurantiacus*.

## 7.2 Introduction

Enzymatic hydrolysis of lignocellulosic biomass to produce fermentable sugars has received much attention for the production of biofuels and high value added biomaterials in biorefineries. Filamentous fungi have long been used for the production of lignocellulose degrading enzymes, especially *Trichoderma reesei* and *Aspergillus niger*, which are used industrially. Industrial enzyme products are, however, expensive and therefore a lot of research has been carried out to lower the cost of the enzymatic conversion of biomass. One approach for this is on-site enzyme production, where a waste stream is used as a substrate for an organism to produce the enzymes at the biorefinery. For this approach it is often beneficial to use crude enzymes, since an expensive downstream processing step is avoided.

*Aspergillus carbonarius* has shown promise as an on-site enzyme production organism [1]. Crude enzymes of *A. carbonarius* were combined with crude enzymes of *T. reesei* Rut-C30, whereby 80 % cellulose conversion efficiency was reached. This approach may be further improved by supplementation with additional cellulose acting enzymes, such as lytic polysaccharide monooxygenases (LPMOs) as this may enhance efficiency and potentially lower the required enzyme load. The effectiveness of LPMOs has been shown on crystalline cellulose using either a non-enzymatic donor of electrons or the cellobiose dehydrogenase (CDH) enzyme [2]. The LPMOs are capable of inserting an oxygen at either C1 or C4 (potentially C6) position of the glycoside bond in cellulose. Type 1 predominantly generates C1 oxidized products and type 2 generates mostly C4, whereas type 3 oxidizes at both reducing and nonreducing ends [3–6].

Cleavage of polysaccharides by C1 oxidation has been proven thoroughly [5,7–12], and the activity on C4 and C6 has been suggested through mass spectrometry analysis [5,6,13]. For instance the *Podospora anserina* GH61 (Pa_LPMO) is believed active on the C6 position of cellulose polymers, while acting in synergy with a CDH from *Pycnoporus cinnabarinus* [13]. It should be noted that cellobiohydrolase (CBH), active on the non-reducing end, benefit more from the C1 oxidation, compared to CBH active on the reducing end, which on the other hand benefit from C4 oxidized breaks. The mixture of oxidative and cellulolytic enzymes generates monomeric and dimeric oxidized sugars. In the case of C1 oxidization it is primarily cellobionic and gluconic acid. Cellobionic acid has been shown to have less inhibitory effect on cellulases and can be hydrolyzed by β-glucosidases to form gluconic acid. Both cellobionic and gluconic acid can be metabolized by fungi [14].

Some LPMOs have also been shown to be active on other substrates than cellulose, for instance hemicellulose and chitin. In the case of *Neurospora crassa* NcLPMO9C it has been shown to be particular

active on the glucose backbone of xyloglucan, accepting various substitutions [15]. The stimulatory effect of LPMO on pretreated corn stover was proven using a combination of *Trichoderma reesei* cellulase mixture and a *Thermoascus aurantiacus* LPMO (Ta_LPMO), resulting in a 1.9 fold reduction of the required protein loading to achieve 91 % cellulose conversion [16]. A C1 oxidizing LPMO from *Thermoascus aurantiacus* was supplemented to *Trichoderma reesei* CBHI in combination with CDH as electron donor. This combination of enzymes improved microcrystalline cellulose hydrolysis by 10 % [2]. It has also been proven that complementation of a cellobiose dehydrogenase (CDH) enzyme to crude enzymes of *Trichoderma reesei* increases overall lignocellulose degradation and produces appreciable amounts of gluconic acid [17]. However, by disruption of two CDH genes from *Myceliophthora thermophila* the crude enzyme hydrolysis of corn stover had a 20 % increased glucose yield, compared to the wild type [18]. In contrast by combining LPMO, CDH and a GH3 β-glucosidase conversion of phosphoric acid swollen cellulose (PASC) to glucose monomers increased by approximately 20 % [19].

In this study the effect of supplementing *A. carbonarius* crude enzyme extract with purified Pa_LPMO and Ta_LPMO was analyzed to determine the enhancing effect in hydrolysis of pretreated wheat straw. The complementing activity was also analyzed by heterologous expression of Pa_LPMO and a CDH from *Neurospora crassa* (Nc_CDH) [20] in *A. carbonarius*.

## 7.3 Materials and methods

### 7.3.1 *Fungal strains*

The study concerns the fungal strain, *A. carbonarius* ITEM 5010, which was maintained in 10 % glycerol at -80 °C. *Pichia pastoris* KM71H (Invitrogen, Carlsbad, CA) was used for transformation and heterologous expression of LPMO and CDH.

### 7.3.2 *Plasmid construction of LPMO and CDH*

The lytic polysaccharide monooxygenase (LPMO) gene GH61B (GenBank accession number: CAP68375.1) from *Podospora anserina S mat+* was codon optimized for production in *A. carbonarius* using the genome of *A. niger* and ordered from GenScript (USA). From *N. crassa* (OR74A) the cellobiose dehydrogenase gene, cdh1 (GenBank accession number: XM_951498.2) was also codon optimized using the genome of *A. niger* and ordered from GenScript (USA) for production in *A. carbonarius*. These genes were USER-cloned to the pPICZα (InvitrogenTM) vector backbone, in line with the multiple cloning site maintaining the native secretion signal of both genes. For primers used in the USER-cloning, see Appendix III (Table A1).

### 7.3.3 *Transformation and screening*

Transformation of competent *P. pastoris* KM71H was performed by electroporation with Bpu1102I linearized pPICZα recombinant plasmids. Transformants were screened on YPDS (10 g/L yeast extract, 20 g/L peptone, 10 g/L dextrose and 10 g/L sorbitol) plates with added zeocin (100 µg/mL). Zeocin resistant transformants were then screened for protein expression using a starter culture of 10 mL YPG (10 g/L yeast extract, 20 g/L Hipolypepton, 10 g/L glycerol in 50 mL Falcon tubes) grown at 30 °C in an orbital shaker (300 rpm) O/N, for inoculation of 200 mL YPG culture. The 200 mL YPG was grown for 2 days and cells were spun down at 5000 x G for 5 min, supernatant was removed and the cells were washed with Milli-Q twice, before re-dissolving in 40 mL YP medium (10 g/L yeast extract, 20 g/L Hipolypepton) with 1 % methanol added for induction of protein production.

### 7.3.4 *Production of recombinant enzymes*

Transformants shown to produce the desired proteins were inoculated into 50 mL falcon tubes containing 10 mL YPG and incubated O/N at 30 °C with 300 rpm shaking. The overnight cultures were used as seed for 2000 mL YPG medium in 5000 mL shake flasks, grown at 30 °C with 300 rpm shaking for 48 hours. The cells were spun down at 5000 x G for 15 min, supernatant was removed and the cells were washed with sterile Milli-Q, the step was repeated twice before re-dissolving the cells in 400 mL YP medium. Methanol (400 µL) was added as inducer of the AOX1 promoter of the pPICZα vector, and the cultures were incubated at 30 °C with 300 rpm shaking for 72 hours. Methanol (400 µL) was added at 0, 24 and 48 hours during cultivation. The supernatant was harvested by spinning down the cells at 1500 x G for 40 min, and the protein content was analyzed with a 12 % SDS-Page gel.

### 7.3.5 *Purification of LPMO and CDH*

Culture supernatant was mixed with 50 % ammonium sulfate and left to precipitate O/N. The resulting supernatant was loaded onto a Phenyl-Sepharose column (ϕ 16 x 100 mm, GE Healthcare UK Ltd., Buckinghamshire, UK) equilibrated with 20 mM sodium acetate and 1 M ammonium sulfate (pH 5.0) that was connected to an Äkta fast performance liquid chromatograph. Elution was performed with a linear reverse gradient of 1 to 0 M ammonium sulfate in sodium acetate 20 mM (pH 5.0). Fractions containing the recombinant enzymes were pooled, concentrated and buffer exchanged to buffer A (50 mM $NaH_2PO_4$ and 50 mM NaCl, pH adjusted to 7.4 with NaOH). The supernatant was further purified using a cobalt chelate His-binding resin column (ϕ 7 x 50 mm, GE Healthcare UK Ltd., Buckinghamshire, UK) that was connected to an Äkta fast performance liquid chromatograph and equilibrated with buffer A. Elution was done with a

gradient of 0 to 50 % buffer B (50 mM $NaH_2PO_4$, 50 mM NaCl and 500 mM imidazole, pH adjusted to 7.4 with NaOH). Fractions containing the peak of interest, were pooled and dialyzed against sodium acetate (20 mM, pH 4.5) using a Vivaspin 20 unit (Sartorius, Germany) with a 10 kDa cutoff at 2500 x G. Purity was evaluated by 12 % SDS-Page gels. The *T. aurantiacus* LPMO was supplied by Novozymes A/S and had been expressed and purified as reported in Harris et al. [16].

### 7.3.6 *Transformation of A. carbonarius*

Genomic DNA (gDNA) was isolated via FastDNA SPIN Kit for soil DNA extraction (MP Biomedicals, USA) from *A. carbonarius* growing on solid standard glucose based minimal medium (MM) (1 % glucose, 1x nitrate salt solution [21], 0.001 % Thiamine, 1x trace metal solution [22], 2 % agar). For transformation medium (TM) glucose was replaced by 1 M sucrose. Plasmids were propagated in *Escherichia coli* DH5α. A gene targeting vector was constructed for insertion of Pa_LPMO and Nc_CDH into the *A. carbonarius albA* locus. Pa_LPMO was put under control of the *A. niger coxA* promoter [23] and the *A. niger trpC* terminator. Nc_CDH was put under control of the *A. nidulans gpdA* promoter and the *A. nidulans trpC* terminator. The two genes with promoter and terminators were placed back to back and together with the hygromycin resistance marker *hph* [24], was flanked by 2kb upstream and downstream sequences for the *A. carbonarius albA* gene. For improved gene targeting the CRISPR-Cas9 system for filamentous fungi developed by Nødvig et al. [25], was used. The protospacer AGTGGGATCTCAAGAACTAC, targeting *albA* was introduced into the vector pFC330, with the finished vector containing the AMA1 element for autonomous replication [26], the *A. fumigatus pyrG* as selection marker, a codon optimized *Cas9* and the guide RNA embedded between two ribozymes. Transformation was performed according to Nødvig et al. [25]. Correct transformants were identified by white spore forming phenotypes on plates containing hygromycin, these were streak purified and verified by PCR with gDNA purified by FastDNA SPIN Kit for soil DNA extraction (MP Biomedicals, USA). See Appendix III for the primers that were used for transformation and check of the mutants.

### 7.3.7 *Spore suspension*

The *A. carbonarius* wildtype and mutants 1 to 6 were grown on minimal medium, and spores were harvested by addition of sterile Milli-Q water with 0.1 % Tween-80. Spore suspensions were filtered through Miracloth (Merck, Millipore), followed by spore counting using a Bürker Türk counting chamber (Hounissen).

### 7.3.8 *Crude enzyme production*

Solid state fermentation (SSF) was used for *A. carbonarius* enzyme production. The solid medium was made in accordance with the procedure reported by Kolasa et al., containing wheat bran (WB) and sphagnum peat [1]. This medium was inoculated with 1 mL of 2 x 106 spores/mL suspension and incubated at 30 °C for 7 days. Flasks were shaken manually twice a day for aeration and equal distribution of the spores. All growth in SSF was set up in quadruplicates. The enzymes were harvested by adding 50 mL sterile Milli-Q, to each flask and shaking them at 4 °C, 200 rpm, overnight. The slush was squeezed through Miracloth (Merck, Millipore) and centrifuged at 4 °C for 20 min at 10,000 x G. The supernatants were transferred to fresh 50 mL Falcon tubes and centrifuged as above. Resulting supernatants were used as crude enzyme extracts in the enzyme assays.

### 7.3.9 *Avicelase activity*

The capacity to hydrolyze avicel was based on determination of total release of reducing sugars by using an Agillent 1100 series high performance liquid chromatography (HPLC) system equipped with an aminex HPX-87H column (Biorad). The assay was carried out using 500 µL 1 % avicel PH-101 (Sigma-Aldrich) in 10 mM sodium acetate (pH 5.0) mixed with 250 µL crude enzyme extract and 250 µL 10 mM sodium acetate pH 5.0 and incubated at 50 °C, 1400 rpm (Eppendorf Thermomixer comfort) for 24 h. The reactions were stopped by boiling them at 100 °C for 10 min, and adding 45 µL 10 % sulfuric acid before spinning down the solids at 16,000 x G for 10 min. The supernatant was collected for HPLC analysis of total amount glucose released. Additionally the boosting effect of Pa_LPMO and Ta_LPMO were analyzed by exchanging the volume of 250 µL sodium acetate buffer with the appropriate volume of the additional enzymes to reach 0.21 and 4.21 mg protein/g cellulose (mg/g) per reaction. This was performed in combination with the electron donor's ascorbate and CDH.

### 7.3.10 *Wheat straw hydrolysis*

Crude enzymes from *A. carbonarius* were analyzed for hydrolysis efficiency of wheat straw (kindly donated by Biogasol ApS, Denmark) which had been pretreated by steam explosion and dilute acid as described by Baroi et al. [27]. The cellulose content of the wheat straw was 36 % and the amount of total solids was 22 % as seen in Table 7.1. In the assays 5 % total solids were applied.

**Table 7.1** Composition of Biogasol steam exploded pretreated wheat straw.

| Structural component | Pre-treated wheat straw % DM |
|---|---|
| **Cellulose** | |
| β-1,4-glucan | **36** |
| **Hemicellulose** | **18.1** |
| Xylan | 16.6 |
| Arabinan | 1.5 |
| **Klason lignin** | **22.1** |
| **Ash** | **1.3** |
| **Residuals** | **22.5** |
| **Total** | **100 %** |

A total volume of 600 µL crude enzyme was added to the pretreated wheat straw in 700 µL 0.1 M succinic acid buffer (pH 5.0) and incubated at 50 °C, 1400 rpm (Eppendorf Thermomixer comfort), for 3 days. After incubation the samples were boiled at 100 °C for 10 min, mixed with 45 µL of 10 % sulfuric acid and centrifuged at 16,000 x G, for 10 min: then the supernatants were analyzed for released sugars using an HPLC equipped with an aminex HPX-87H column (Biorad).

Synergy tests were performed by supplementing 600 µL crude enzyme extract with defined amounts of purified proteins added at either 0.21 or 4.21 mg/g of Pa_LPMO in combination with 0.42 mg/g of Nc_CDH. Additionally L-ascorbic acid sodium salt was analyzed for the efficiency as electron donor, applied at a final concentration of 10 mM instead of 0.42 mg/g CDH. As a positive control, the purified *T. aurantiacus* LPMO (AA9) from Novozymes A/S (Ta_LPMO) was applied at a final concentration of either 0.21 or 4.21 mg/g, as supplement for 600 µL crude enzymes with the two different electron donors Nc_CDH (0.42 mg/g) or ascorbate (10 mM).

Finally Celluclast 1.5 L and Novozyme 188 were analyzed for synergy with the purified LPMO's. Celluclast 1.5 L and Novozym 188 were applied at a 25 times diluted concentration in an optimal ratio of 4:1 (40 µL and 10 µL respectively) corresponding to 0.0016 U/mL avicelase, 0.156 U/mL endoglucanase and 0,096 U/mL β-glucosidase activities in the reactions. The quadruplicate enzyme extract samples were tested in duplicate hydrolysis reactions.

## 7.4 Results and Discussion

**Heterologous expression of Pa_LPMO and Nc_CDH in *P. pastoris***. The LPMO enzyme from *Podospora anserina* (Pa_LPMO) was chosen because of the already proven activity on phosphoric acid swollen cellulose (PASC) with the use of a class I-CDH from *Pycnoporus cinnabarinus* as electron donor [13]. The CDH from *N. crassa* (Nc_CDH) falls within the class II distinction based on sequence similarity, however, the enzymatic properties resemble class-I CDH, which are more common in basidiomycetes [20]. Both enzymes

were predicted to have a carbohydrate binding module 1 (CBM 1), which ensures binding to cellulose. The combination of Nc_CDH and Pa_LPMO has not been analyzed previously. The cDNA encoding Nc_CDH and Pa_LPMO was codon optimized towards *A. niger* and were inserted into pPICZα for expression in *Pichia pastoris*. Both enzymes were expressed successfully and the purified enzymes displayed apparent masses of 50 kDa (36 kDa predicted) and 95 kDa (85 kDa predicted) for Pa_LPMO and Nc_CDH, respectively (see Appendix IV, lane 2 and 4). These higher values result in part from the c-myc epitope and the $His_6$-tag at the N-terminus, but majority of the weight likely stems from N- and O-glycosylation. The weight of the *T. aurantiacus* LPMO, is 28 kDa. This difference in size between Pa_LPMO and Ta_LPMO is in part because of the lack of a CBM module in the Ta_LPMO but also due to different expression hosts.

**Avicel hydrolysis**. To further investigate the cellulose degrading activity of the Pa_LPMO and Nc_CDH, the enzymes were supplemented to a crude enzyme extract of *A. carbonarius* for 24 h hydrolysis of crystalline cellulose in the form of avicel. Fig. 7.1 shows that no significant increase in the released glucose could be seen when the Pa_LPMO was supplemented to the crude enzymes of *A. carbonarius* at both a low (0.21 mg/g) and a high concentration (1.98 mg/g) with the electron donors ascorbate (10 mM) and CDH (0.42 mg/g). Further there was no observed increase by supplementing with Ta_LPMO at a low concentration (0.21 mg/g), regardless of the electron donor supplied. It may be an effect of the low concentration of Ta_LPMO that an unchanged glucose release was observed compared to the control, since there was a boosting effect when a high concentration was applied. For Ta_LPMO (4.21 mg/g) in combination with ascorbate (10 mM) the total released glucose was increased by 0.15 g/L to a total of 0.48 g/L compared to the control *A. carbonarius* without supplement 0.33 g/L. However, the effect of the high concentration of Ta_LPMO was not observed when it was supplemented in combination with Nc_CDH (0.42 mg/g), where the glucose release was 0.30 ± 0.02 g/L which is within the standard deviation of the control 0.33 g/L ± 0.03 g/L.

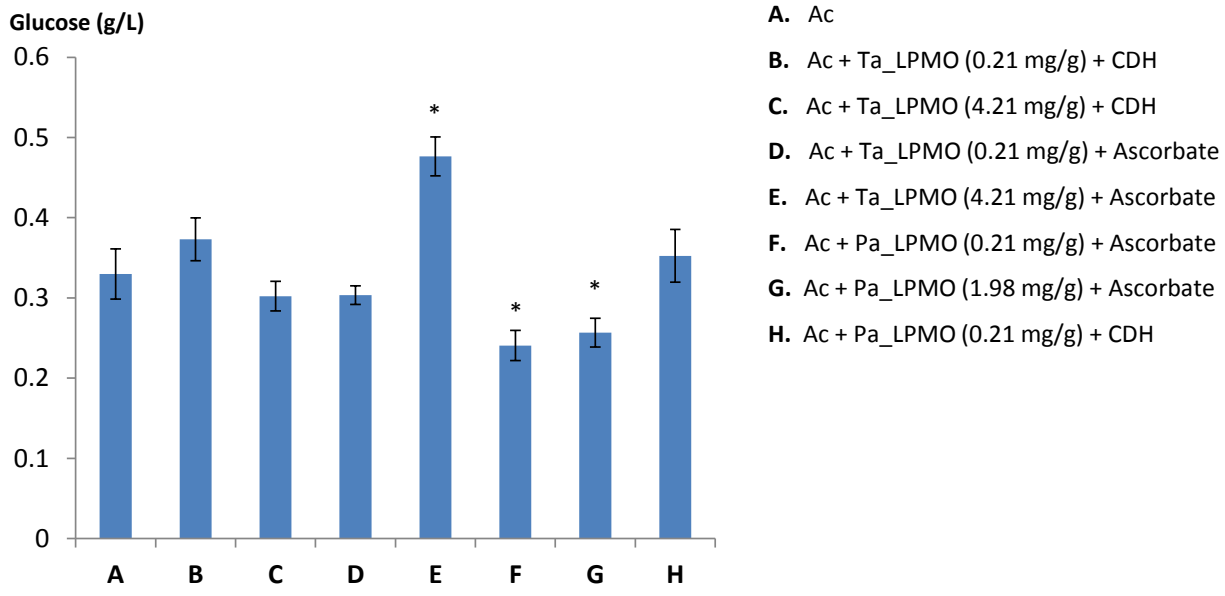


**Figure 7.1** Glucose release from avicel hydrolysis. A) Crude enzymes of *A. carbonarius* (Ac), B – H) Supplementation with both Pa_LPMO and Ta_LPMO to Ac. Error bars denote standard deviations (SD) (N = 3). *, P < 0.05 (Student t test)

The decreasing effect of the added CDH enzyme may be a consequence of either inefficient electron donor capacity or a decrease in the accessibility of the cellobiose for the *A. carbonarius* β-glucosidases. Previously it has also been observed that application of a *Podospora anserina* CDH (0.42 mg/g) to a commercial enzyme preparation from *Trichoderma reesei* CL847 reduced the glucose released by 30 %, but increased the amount of gluconic acid [28]. In this study the glucose and gluconic acid fraction were co-eluting, when using the aminex HPX-87H column, meaning that the total glucose measured contains the amount of gluconic acid produced. However, the previously observed decrease in glucose when supplementing *P. anserina* CDH (0.42 mg/g), from 2100 µM glucose to 1150 µM was not balanced by the corresponding production of 200 µM gluconic acid [28]. This provides one explanation for the reduced glucose release found in this study, when supplementing with Ta_LPMO (4.21 mg/g) and CDH (0.42 mg/g) compared to applying 10 mM ascorbate in combination with Ta_LPMO (4.21 mg/g). The addition of CDH to a high concentration of Pa_LPMO (1.98 mg/g) was not assayed due to the lacking effect when supplementing to the high concentration of Ta_LPMO.

**Hydrolysis of wheat straw.** To test the application potential of the purified Pa_LPMO and Nc_CDH when supplementing a crude enzyme mixture, hydrolysis of steam pretreated wheat straw was performed. Again it was analyzed whether LPMO from *Podospora anserina* could boost the hydrolysis potential of *A. carbonarius* crude enzymes. Two concentrations were analyzed, 0.21 and 4.21 mg/g in combination with different electron donors; the purified CDH from *N. crassa* (0.42 mg/g) and ascorbate (10 mM). From this it

is apparent that when applied at 0.21 mg/g, the *Pa*_LPMO cannot boost hydrolysis of wheat straw of *A. carbonarius* crude enzymes, there even appears to be a small decline in the released glucose (Fig. 7.2). When supplementing with *Pa*_LPMO and *Nc*_CDH, the glucose released is at 5.70 ± 0.19 g/L compared to the crude enzymes alone releasing 5.91 ± 0.10 g/L. Even with addition of ascorbate as alternative electron donor no apparent boosting could be observed when applying *Pa*_LPMO at 0.21 mg/g (Fig. 7.2).

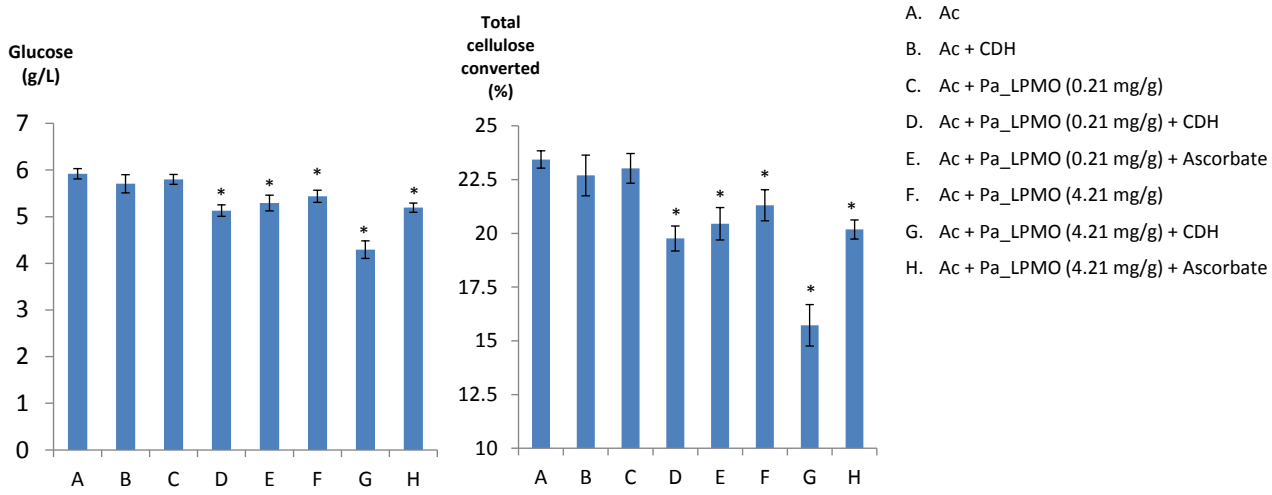


**Figure 7.2** Wheat straw hydrolysis glucose release (left) and effeciency of hydrolysis (right). A) *A. carbonarius* (Ac) crude enzymes, and for B – H) supplementing with Pa_LPMO. Error bars denote SD (N = 3). *, P < 0.05 (Student t test)

When *Pa*_LPMO was applied at 4.21 mg/g with the same electron donors, no increasing effect on glucose release could be observed either. Previously the *Pa*_LPMO was shown to be active on phosphoric acid swollen cellulose by measurement of different oxidized cello-oligosaccharides, and it was also produced in *Pichia pastoris* using pPICZα vector [13]. The missing effect might be caused by application of too low concentrations of Pa_LPMO. It is unlikely that glycosylation or the HIS-tag affects the protein activity since the same expression host (*P. pastoris*) and vector was used previously [13]. It is apparent that there was a significant decrease of glucose when 4.21 mg/g Pa_LPMO and Nc_CDH was supplemented to the hydrolysis reaction. This could be either an effect of reduced buffering in the reaction mixture, or because of the cellulose substrate being blocked by the binding of CBM from Pa_LPMO and from Nc_CDH. The CBM module is the main difference between Pa_LPMO and the Ta_LPMO which does not contain one.

Mutants of *A. carbonarius* were made for constitutive expression of Pa_LPMO and Nc_CDH, in order to determine whether an improvement of the crude enzymes could be achieved in this way compared to supplementing with purified Pa_LPMO and Nc_CDH produced in *P. pastoris*. The crude enzymes of all six mutants, were analyzed by hydrolysis of wheat straw (Fig. 7.3). Based on the glucose release it is clear that

there was a slight, but statistically significant ($P < 0.05$), improvement of glucose release to 6.36 g/L observed for mutant 1 compared to wildtype having a 5.92 g/L glucose release. This might be because of a higher expression of the Pa_LPMO in mutant 1. All other five mutant strains (Ac mutant 2-6), did not show any improvement of the glucose release compared to the wildtype. These results could be a sign of either low protein secretion or incorrect folding in *A. carbonarius*. However, in combination with the results observed for supplementing with purified Pa_LPMO and Nc_CDH from *P. pastoris* it indicates that these two enzymes cannot be applied as efficient boosters of wheat straw hydrolysis with regards to improved glucose release for *A. carbonarius*.

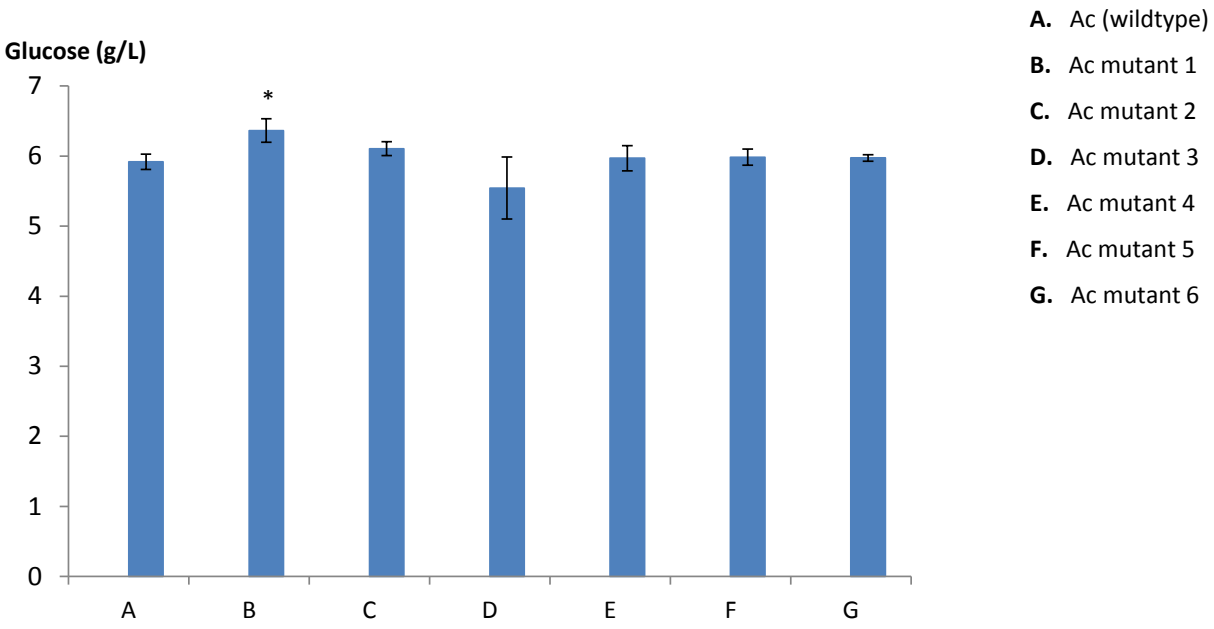


**Figure 7.3** Glucose release from wheat straw hydrolysis. A) Crude enzymes from *A. carbonarius* (Ac (wildtype)), and six Pa_LPMO + Nc_CDH mutants (B - G). Error bars denote SD (N = 3). *, $P < 0.05$ (Student t test)

Interestingly there was a statistically significant increase of xylose released in the wheat straw hydrolysis, observed for all the mutants except no. 3. This was also obtained for supplementing the crude enzymes with Ta_LPMO (Fig. 7.4). Mutant 6 showed the highest increase of xylose by 1.2 g/L compared to the wildtype. For the Ta_LPMO, an increase in xylose of 0.81 g/L was also observed and in general the transformants 1, 2, 4 and 5 had an average higher xylose release of 0.83 g/L as seen in Fig. 7.4. It has previously been shown that an LPMO from *Neurospora crassa* (*NcLPMO9C*) was primarily active towards the glucose backbone of xyloglucan [15]. This may indicate that both the Pa_LPMO and the Ta_LPMO analyzed in this study also show activity towards xyloglucan. It could also be an effect of the oxidative activity towards the cellulose structure whereby accessibility to the xylan backbone increases. Recently it was also found that a C1 and C4 oxidizing LPMO of the AA9 class from *Myceliopthora thermophila* C1 could

oxidize xylan-oligosaccharides and cellulose in the presence of ascorbate [29]. Also a recent study of seven *P. anserina* LPMO activities, which omitted the LPMO studied here, showed that PaLPMO9H had a broad substrate specificity including xyloglucans [30]. Therefore it is plausible that the Pa_LPMO analyzed here is primarily active on xyloglucans, which explains the increased xylose release observed for the mutants.

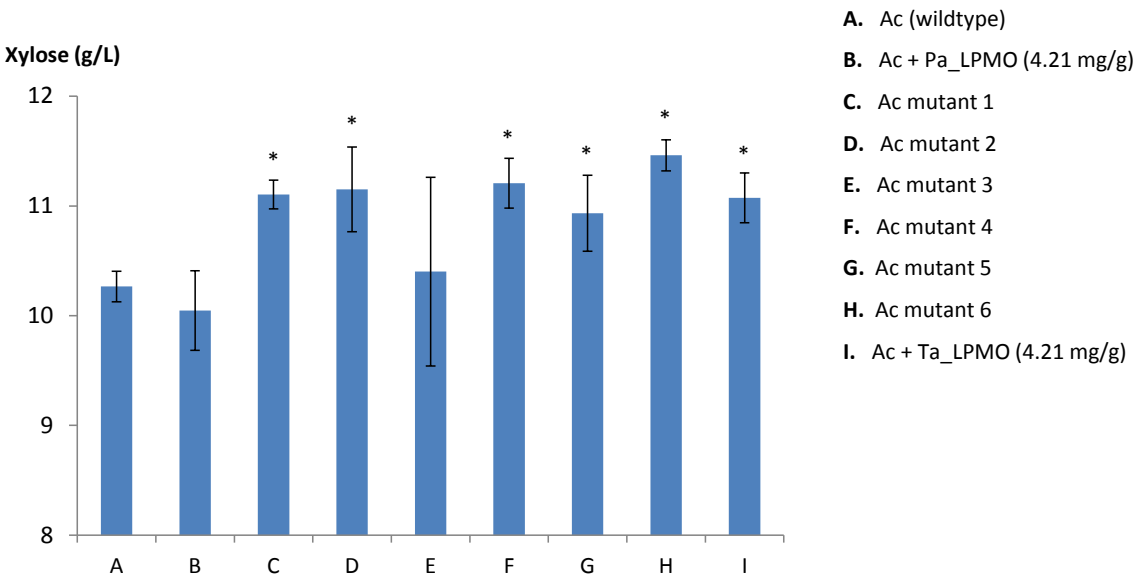


**Figure 7.4** Xylose release from wheat straw hydrolysis. A) crude enzymes from *A. carbonarius* (Ac (wildtype)), and six Pa_LPMO + Nc_CDH mutants (C – H). Also purified Pa_LPMO (B) and Ta_LPMO (I) was supplemented. Error bars denote SD (N = 3). *, $P < 0.05$ (Student t test)

Analysis of *A. carbonarius* whole genome sequence (JGI, Aspca3) using dbCan to annotate the proteins and CDD as well as interPro to check the annotations, reveals that it contains three genes coding for LPMO's (AA9) (amino acid sequences are presented in Appendix V). A secretome analysis of *A. carbonarius* grown on WB showed that two of the predicted three genes of LPMOs were expressed (data not published), and it is expected that under the cultivation conditions applied in this study these two LPMO's should also be expressed. The two *A. carbonarius* LPMO's are apparently not expressed in a sufficient amount or they have different substrate specificities, because supplementing with 0.21 mg/g of the purified *T. aurantiacus* LPMO to *A. carbonarius* crude enzymes resulted in a significant boosting effect of the wheat straw hydrolysis (Fig. 7.5).

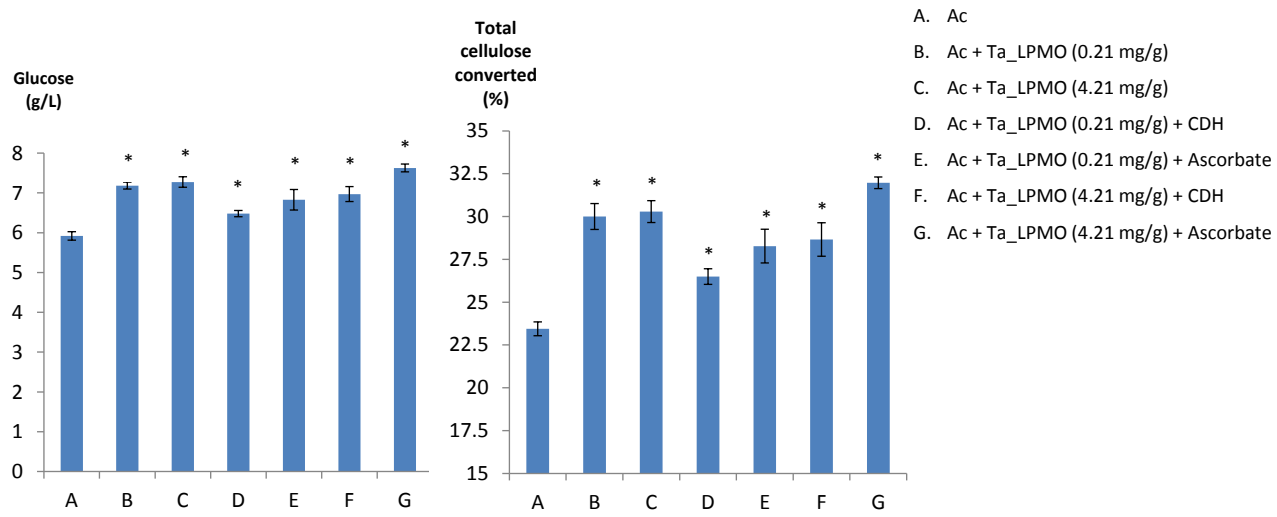


**Figure 7.5** Wheat straw hydrolysis glucose release (left) and effeciency of hydrolysis (right). A) *A. carbonarius* (Ac) crude enzymes, and B - G) supplementing with Ta_LPMO. Error bars denote SD (N = 3). *, P < 0.05 (Student t test)

Since it was possible to boost hydrolysis by supplementing with the Ta_LPMO, there must either be a low expression of *A. carbonarius* own LPMO's or the genes are not induced by the WB/SP medium used for cultivation. The effect was obtained without adding an electron donor to the reaction mixture, indicating that either the *A. carbonarius* crude enzyme extract or the pretreated wheat straw contains an electron donor that can efficiently supply the Ta_LPMO. Previously it has been shown that LPMO's can function effectively without the known electron donors such as ascorbate, gluthathione, gallic acid or CDH, instead utilizing lignin or aldonic acids [31,32]. Furthermore the pretreatment method used for the lignocellulose influence the reactivity of the lignin towards LPMO, it was found that hydrothermal pretreatment of corn stover, sugarcane bagasse and wheat straw resulted in better preservation of the reactivity compared to organosolvent or alkaline techniques [33]. The pretreated wheat straw applied in this study contains 22.1 % klason lignin, which could explain the observed boosting activity of the Ta_LPMO applied at both 0.21 and 4.21 mg/g without additional electron donors (Fig. 7.5). Without adding an external electron donor, an increase of 1.26 g/L was observed for supplementing with 0.21 mg/g and a 1.35 g/L increase when applying 4.21 mg/g Ta_LPMO, compared to using purely *A. carbonarius* crude enzymes. In addition supplementing with the electron donor ascorbate (10 mM) can facilitate a statistically significant (P < 0.05) higher glucose release of 7.63 g/L compared to relying on the lignin reactivity where a glucose release of 7.27 g/L was observed for application of 4.21 mg/g Ta_LPMO. This corresponds to a hydrolysis efficiency increase of 6.8 % when relying on lignin or 8.5 % when adding ascorbate in combination with 4.21 mg/g Ta_LPMO. This was, however, not the case for ascorbate applied to reactions with 0.21 mg/g Ta_LPMO indicating that

when using 4.21 mg/g protein load, lignin alone may not be sufficient to support the high concentration of LPMO.

To further test the application potential of the purified LPMO's from *Podospora anserina* and *T. aurantiacus*, they were both added to hydrolysis performed with Celluclast 1.5 L and Novozym 188. For the Ta_LPMO hydrolysis efficiency increased to 68.03 ± 1.95 %, compared to the Celluclast 1.5 L and Novozym 188 efficiency of 42.85 ± 1.74 %. The single addition of Ta_LPMO could improve the hydrolysis significantly by 25.19 % (Fig. 7.6), again without additional electron donor supplied. Interestingly the Ta_LPMO is 17 % more beneficial when supplemented to Celluclast 1.5 L and Novozym 188, compared to *A. carbonarius* crude enzymes. This shows that the efficiency of the LPMO boosting effect is also dependent on the amount and type of cellulases and β-glucosidases present in the reaction mixture. Therefore, for application of *A. carbonarius* crude enzymes in lignocellulose hydrolysis, an additional improvement would be needed to increase the amount of cellobiohydrolases and endoglucanases. Application of LPMO from *P. anserina* showed no increase in the overall glucose released when supplied to Celluclast 1.5 L and Novozym 188 at a protein load of 0.21 mg/g.

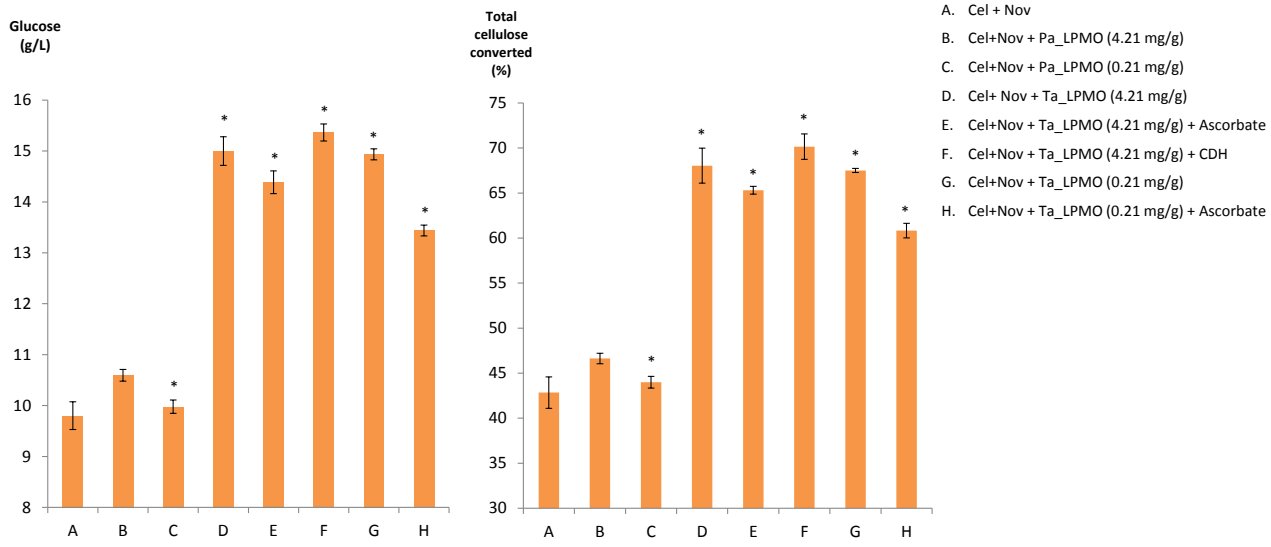


**Figure 7.6** Glucose release from wheat straw hydrolysis (left) and efficiency of hydrolysis (right) for A) Celluclast 1.5 L (Cel) and Novozym 188 (Nov), and B – H) supplementation with Pa_LPMO and Ta_LPMO. Error bars denote SD (N = 3). *, $P < 0.05$ (Student t test)

However, when 4.21 mg/g Pa_LPMO was applied the glucose release increased to 10.59 ± 0.12 g/L, compared to the Cel + Nov reaction having a glucose release of 9.80 ± 0.27 g/L. This indicates that the Pa_LPMO does have a small but significant ($P < 0.05$) influence on the glucose release, when applied at a high concentration and in combination with an efficient mixture of cellulases, presumably

cellobiohydrolases in particular, which are not very efficient in *A. carbonarius*. Also in the previous study of Pa_LPMO the enzyme was applied at a 5 and 50 times higher protein loading, which could explain why they observed oxidized products in PASC hydrolysis [13]. It furthermore seems very clear that the Ta_LPMO being a C1 – C4 oxidizing enzyme compared with the C6-oxidizing Pa_LPMO, is much more efficient for synergistically improving hydrolysis together with the commercial cellulase mixture.

## 7.5 Conclusion

Crude enzymes of *A. carbonarius* were applied for hydrolysis of wheat straw with an efficiency of 23 %. This study showed that the addition of a single enzyme, Ta_LPMO, can boost the efficiency by 6.8 % when applied at 4.21 mg/g without additional electron donor. Even at a low concentration (0.21 mg/g) the efficiency could be increased by 6.6 %. At the high concentration of LPMO (4.21 mg/g) the hydrolysis efficiency could be further increased by 1.7 % when supplementing with ascorbate (10 mM). Pa_LPMO and Nc_CDH were expressed in *A. carbonarius*, and the resulting crude enzymes showed an increase of 0.83 - 1.2 g/L xylose from wheat straw hydrolysis.

## 7.6 Acknowledgements

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## 7.7 References


[1] Kolasa M, Ahring BK, Lübeck PS, Lübeck M. Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. Bioresour Technol 2014;169:143–8.

[2] Langston JA, Shaghasi T, Abbate E, Xu F, Vlasenko E, Sweeney MD. Oxidoreductive cellulose depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61. Appl Environ Microbiol 2011;77:7007–15.

[3] Isaksen T, Westereng B, Aachmann FL, Agger JW, Kracher D, Kittl R, et al. A C4-oxidizing lytic polysaccharide monooxygenase cleaving both cellulose and cello-oligosaccharides. J Biol Chem 2014;289:2632–42.

[4] Beeson WT, Phillips CM, Cate JHD, Marletta MA. Oxidative cleavage of cellulose by fungal copper-dependent polysaccharide monooxygenases. J Am Chem Soc 2012;134:890–2.

[5] Phillips CM, Beeson WT, Cate JH, Marletta MA. Cellobiose dehydrogenase and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by *Neurospora crassa*. ACS Chem Biol 2011;6:1399–406.

[6] Quinlan RJ, Sweeney MD, Leggio L Lo, Otten H, Poulsen J-CN, Johansen KS, et al. Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. Proc Natl Acad Sci U S A 2011;108:15079–84.

[7] Forsberg Z, Vaaje-kolstad G, Westereng B, Bunsæ AC, Stenstrøm Y, Mackenzie A, et al. Cleavage of cellulose by a cbm33 protein. Protein Sci 2011;20:1479–83.

[8] Hemsworth GR, Taylor EJ, Kim RQ, Gregory RC, Lewis SJ, Turkenburg JP, et al. The copper active site of CBM33 polysaccharide oxygenases. J Am Chem Soc 2013;135:6069–77.

[9] Vaaje-Kolstad G, Westereng B, Svein JH, Zhanliang L, Hong Z, Sørlie M, et al. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 2010;330:219–22.

[10] Westereng B, Agger JW, Horn SJ, Vaaje-Kolstad G, Aachmann FL, Stenstrøm YH, et al. Efficient separation of oxidized cello-oligosaccharides generated by cellulose degrading lytic polysaccharide monooxygenases. J Chromatogr A 2013;1271:144–52.

[11] Westereng B, Ishida T, Vaaje-Kolstad G, Wu M, Eijsink VGH, Igarashi K, et al. The putative endoglucanase pcGH61D from *Phanerochaete chrysosporium* is a metal-dependent oxidative enzyme that cleaves cellulose. PLoS One 2011;6.

[12] Wu M, Beckham GT, Larsson AM, Ishida T, Kim S, Payne CM, et al. Crystal structure and computational characterization of the lytic polysaccharide monooxygenase GH61D from the basidiomycota fungus *Phanerochaete chrysosporium*. J Biol Chem 2013;288:12828–39.

[13] Bey M, Zhou S, Poidevin L, Henrissat B, Coutinho PM, Berrin J-G, et al. Cello-oligosaccharide oxidation reveals differences between two lytic polysaccharide monooxygenases (family GH61) from *Podospora anserina*. Appl Environ Microbiol 2013;79:488–96.

[14] Fan Z, Wu W, Hildebrand A, Kasuga T, Zhang R, Xiong X. A novel biochemical route for fuels and chemicals production from cellulosic biomass. PLoS One 2012;7:1–8.

[15] Agger JW, Isaksen T, Várnai A, Vidal-Melgosa S, Willats WGT, Ludwig R, et al. Discovery of LPMO activity on hemicelluloses shows the importance of oxidative processes in plant cell wall degradation. Proc Natl Acad Sci USA 2014;111:6287–92.

[16] Harris PV., Welner D, McFarland KC, Re E, Navarro Poulsen JC, Brown K, et al. Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: Structure and function of a large, enigmatic family. Biochemistry 2010;49:3305–16.

[17] Bey M, Berrin J-G, Poidevin L, Sigoillot J-C. Heterologous expression of *Pycnoporus cinnabarinus* cellobiose dehydrogenase in *Pichia pastoris* and involvement in saccharification processes. Microb Cell Fact 2011;10:113.

[18] Emalfarb M, Sinitsyn A, Jundzil R, Wery JJ, Visser R, Joosten M, et al. Method of improving the activity of cellulase enzyme mixtures in the saccharification (ligno)cellulosic material. US 2013/0280764 A1, 2013.

[19] Langston JA, Brown K, Xu F, Borch K, Garner A, Sweeney MD. Cloning, expression, and characterization of a cellobiose dehydrogenase from *Thielavia terrestris* induced under cellulose growth conditions. Biochim Biophys Acta, Proteins Proteomics 2012;1824:802–12.

[20] Zhang R, Fan Z, Kasuga T. Expression of cellobiose dehydrogenase from *Neurospora crassa* in *Pichia pastoris* and its purification and characterization. Protein Expr Purif 2011;75:63–9.

[21] Kaminskyj S. Fundamentals of growth, storage, genetics and microscopy of *Aspergillus nidulans*. Fungal Genet Newsl 2001:25–31.

[22] Cove DJ. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochim Biophys Acta 1966;113:51–6.

[23] Blumhoff ML, Steiger MG, Mattanovich D, Sauer M. Targeting enzymes to the right compartment: Metabolic engineering for itaconic acid production by *Aspergillus niger*. Metab Eng 2013;19:26–32.

[24] Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, Hondel CAMJJ Van Den. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 1987;56:117–24.

[25] Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH. A CRISPR-Cas9 System for Genetic Engineering of Filamentous Fungi. PLoS One 2015;10:e0133085.

[26] Gems D, Johnstone IL, Clutterbuck a. J. An autonomously replicating plasmid transforms *Aspergillus nidulans* at high frequency. Gene 1991;98:61–7.

[27] Baroi GN, Skiadas I V., Westermann P, Gavala HN. Continuous Fermentation of Wheat Straw Hydrolysate by *Clostridium tyrobutyricum* with In-Situ Acids Removal. Waste and Biomass Valorization 2015;6:317–26.

[28] Turbe-Doan A, Arfi Y, Record E, Estrada-Alvarado I, Levasseur A. Heterologous production of cellobiose dehydrogenases from the basidiomycete *Coprinopsis cinerea* and the ascomycete *Podospora anserina* and their effect on saccharification of wheat straw. Appl Microbiol Biotechnol 2013;97:4873–85.

[29] Frommhagen M, Sforza S, Westphal AH, Visser J, Hinz SWA, Koetsier MJ, et al. Discovery of the combined oxidative cleavage of plant xylan and cellulose by a new fungal polysaccharide monooxygenase. Biotechnol Biofuels 2015;8:101.

[30] Bennati-granier C, Garajova S, Champion C, Grisel S, Haon M, Zhou S, et al. Substrate specificity and regioselectivity of fungal AA9 lytic polysaccharide monooxygenases secreted by *Podospora anserina*. Biotechnol Biofuels 2015:1–14.

[31] Dimarogona M, Topakas E, Olsson L, Christakopoulos P. Lignin boosts the cellulase performance of a GH-61 enzyme from Sporotrichum thermophile. Bioresour Technol 2012;110:480–7.

[32] Cannella D, Hsieh CC, Felby C, Jørgensen H. Production and effect of aldonic acids during enzymatic hydrolysis of lignocellulose at high dry matter content. Biotechnol Biofuels 2012;5:1–10.

[33] Rodriguez-Zúñiga UF, Cannella D, Giordano RDC, Giordano RDLC, Jørgensen H, Felby C. Lignocellulose pretreatment technologies affect the level of enzymatic cellulose oxidation by LPMO. Green Chem 2015;17:2896–903.

# Chapter 8

# Conclusion and Perspectives

In this chapter the Ph.D. study is concluded and results are discussed for future research and application perspectives.

## 8.1 Conclusion

The main goal of this Ph.D. study was to enhance the crude enzymes of *Aspergillus carbonarius* for lignocellulose hydrolysis. The improved lignocellulose hydrolysis would have a valuable application in conversion of garden park waste for sustainable production of organic acids or hydrocarbon fuels. The approaches applied were: blending crude enzymes from monocultures of ascomycetes, optimization of the cultivation method and process, supplementing with purified enzymes and heterologous enzyme expression.

For the first approach several ascomycetes isolated from different relevant environmental niches were screened to use their monoculture crude enzymes for blending with *A. carbonarius*. The analyzed fungi belonged to the genera *Aspergillus*, *Chaetomium*, *Cladosporium*, *Fusarium*, *Gliocladium*, *Penicillium*, *Stachybotrys* and *Trichoderma*. The isolates were cultivated on wheat bran (WB) agar medium and a liquid medium containing wheat bran from which enzymes were extracted and applied as boosters in wheat straw hydrolysis. Blending of crude enzymes from solid cultivations showed that the eight isolates *A. niger, P. allii*, *P. olsonii*, *P. polonicum, P. coprophilum, P. vulpinum* and *Gliocladium* sp. 1 and 2 could significantly boost the glucose release in wheat straw hydrolysis when combined with *A. carbonarius*. Contrastingly, the cultivations in a submerged medium showed that only *Fusarium poae*, *F. graminearum* and *Gliocladium* sp. 1 could boost the glucose release from wheat straw hydrolysis. Furthermore, the submerged cultivation resulted in an enzyme blend that had a higher activity resulting in more glucose released. In the dedicated screening of six different enzyme activities for common indoor fungi a general high activity of endo-xylanase was found for all 21 isolates grown in WB/SP medium. It was also found that two of the isolates showed a higher activity for all analyzed enzymes, these were *P. chrysogenum* and *C. sphaerospermum*. Both isolates could be applied as producers of crude enzymes for boosting *A. carbonarius*.

Optimization of *A. carbonarius* cultivation method for improved cellulase production was analyzed mainly in solid state fermentation, as this approach was hypothesized to yield highest enzyme activities based on literature studies. Media based on the lignocellulosic garden and park waste (GPW) were analyzed for their potential in yielding highest enzyme activities. It was found that the garden and park waste collected by Solum A/S could be applied as a cultivation medium for *A. carbonarius* resulting in a crude enzyme blend that has an efficient hydrolysis of wheat straw yielding a glucose conc. of 5.9 g/L. This glucose release was obtained from crude enzymes of GPW/N and was the highest compared to GPW/MW and WB/L. The nitrogen source was shown to have a significant effect on the cellulase production for both *A. carbonarius* and *T. reesei* Rut-C30. Applying municipal waste as additional nitrogen source in the GPW medium did not improve the medium, but by supplementing with urea and $(NH_4)_2SO_4$ an improved lignocellulose hydrolysis

was observed. Therefore if the GPW is to be applied as an on-site enzyme production medium it is required that the additional nitrogen $(NH_4)_2SO_4$ and urea are supplemented. Furthermore in a WB/SP medium the β-glucosidase activity of *A. carbonarius* was higher than in the GPW/N medium especially with prolonged cultivation time reaching a max of 17 U/ml.

The cultivation of *A. carbonarius* in SSF requires indirect measurements of the biomass to determine growth characteristics. During this Ph.D. study two methods were applied and evaluated for measuring fungal biomass in an SSF system. The biomass accumulation of *A. carbonarius* in a WB/SP medium was analyzed and the method that was most reproducible and reliable was measurements of ergosterol content. Although the method takes longer than measurements of NAHA the standard deviation was much lower for ergosterol ranging from 2 – 8 % compared to NAHA which had 11 – 23 %. Therefore for future determinations of *A. carbonarius* biomass in SSF, ergosterol should be the preferred choice. No linear correlation between measured Erg (biomass) and enzyme activities was found.

Finally it was shown that the hydrolysis potential of *A. carbonarius* crude enzymes could be optimized with regards to increased glucose release from pretreated wheat straw by application of a purified LPMO. The enhancing effect was not dependent on addition of electron donors, due to the presence of lignin in the pretreated wheat straw. Choice of LPMO significantly affected the improvement observed, as there was no glucose increase observed when applying an LPMO from *Podospora anserina*, compared to an LPMO from *Thermoascus aurantiacus*. An improvement of 6.8 % higher cellulose conversion efficiency was achieved when the crude enzymes of *A. carbonarius* was supplemented with a high concentration 4.21 mg/g cellulose of *T. aurantiacus* LPMO (Ta_LPMO). It is though possible to achieve very similar enhancement of the cellulose conversion efficiency, 6.6 %, by using only a small amount of Ta_LPMO, 0.21 mg/g cellulose. It is therefore apparent that a significant improvement of the hydrolysis potential of *A. carbonarius* can be achieved at a low cost due to the requirement of only 0.21 mg/g. It was also shown that it is possible to heterologously express an LPMO from *Podospora anserina* in *A. carbonarius*. This expression resulted in improved xylose release in the wheat straw hydrolysis, and it proved the concept that *A. carbonarius* as an organism can be augmented through metabolic engineering to express LPMO's. In this study it was furthermore presented that the efficiency of the Ta_LPMO supplement also depends on the amount of cellulase and β-glucosidase. It was shown that by supplementing the low concentration (0.21 mg/g cellulose) to Novozym 188 and Celluclast 1.5 L an improvement of the cellulose conversion equivalent to 24.9 % was achieved. This shows the potential of not only supplementing LPMO to *A. carbonarius* but also supplementing with additional cellobiohydrolase and endoglucanase.

This improvement of hydrolysis can be applied for *A. carbonarius* cultivation in a GPW/N medium, to increase growth and thereby production of enzymes and potentially organic acids in a sustainable process using wastes as substrate.

## 8.2 Perspectives of: Screening for cellulase producers and boosters of lignocellulose hydrolysis in different environmental niches

In addition to the results presented in this study there are several experiments that could be performed to further understand the complementarity of the identified boosters as well as optimize the achieved synergy in lignocellulose hydrolysis. The fungal strains that could enhance *A. carbonarius* lignocellulose hydrolysis both based on the WB agar plug extracts and the shake flask cultivations, should be co-cultivated with *A. carbonarius* on the same media. This should be done in order to see whether the interaction between the two fungi could improve the resulting enzyme blend. Also co-cultivation may be more economical feasible for an on-site enzyme production application, since only one reactor would be needed for cultivation instead of two separate reactors. The efficiency of the crude enzymes from co-cultivations should be analyzed by hydrolysis of pretreated wheat straw, for comparison. An initial screening of *A. carbonarius* compatibility for co-cultivation showed that inoculation of any of the *Fusarium* spp. should be done with a delay relative to *A. carbonarius* since all Fusarium spp. had a higher growth rate on WB, see Fig. 8.1 (A - F).

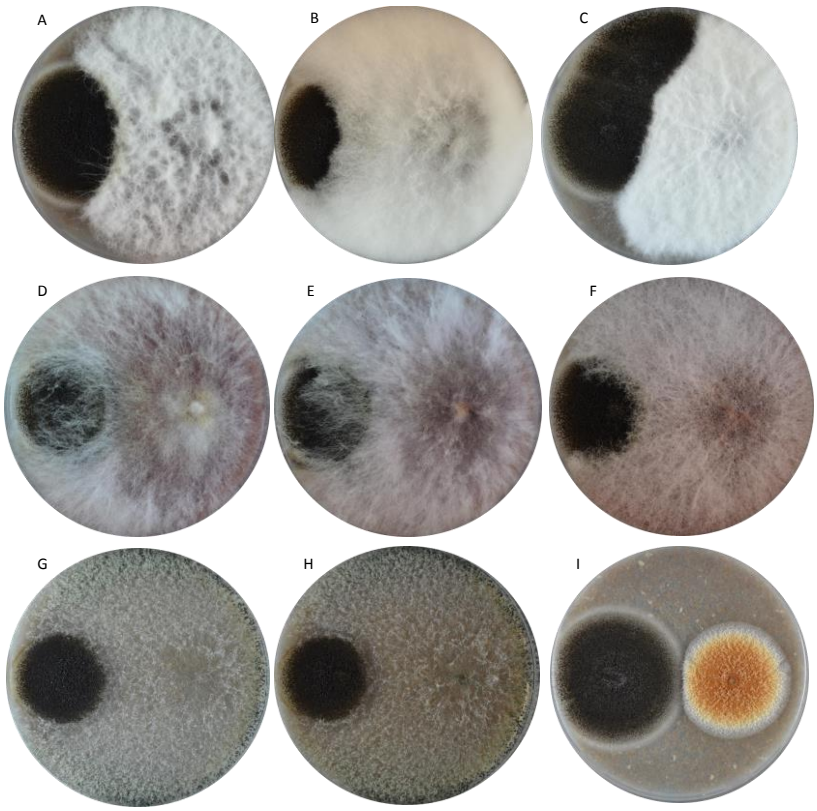


**Figure 8.1** Co-cultivations (7 days) of *A. carbonarius* inoculated on WB-agar medium equidistant and at the same time as, A) *F. poae* 1, B) *F. poae* 2, C) *F. poae* 3, D) *F. culmorum*, E) *F. graminearum*, F) *F. sporotrichioides*, G) *Gliocladium* sp. 1, H) *Gliocladium* sp. 2, I) *Paecilomyces* sp..

These cultivations also show that the most promising boosting organism *Gliocladium* sp. 1, would overgrow *A. carbonarius* in a WB based medium, therefore this fungi also needs to be inoculated with a delay. One of the other fungi analyzed for co-cultivation potential was *Paecilomyces* sp. which had a growth rate similar to that of *A. carbonarius.* The *Paecilomyces* sp. is an example of a fungal species that could be inoculated at the same time point as *A. carbonarius*.

Furthermore, it would be beneficial to cultivate the strains identified in a more optimal medium than the ones applied in this study, such as the WB/SP medium applied by Kolasa et al. [1]. Medium optimization may lead to much higher efficiencies, and therefore it would be beneficial to analyze several media and cultivation setups. As described in chapters 4 and 5 there are large differences in the enzyme activities obtained by the same species whether they are cultivated in solid state or submerged fermentation.

The results in this chapter further showed that it was possible to isolate novel fungal species from relevant environmental niches to be applied as boosters of *A. carbonarius* lignocellulose hydrolysis. Especially the indoor fungal isolates showed promise in the solid state WB-agar cultivations, which therefore led to a

dedicated screening of several indoor fungal isolates. The results of the dedicated screening were presented in chapter 3.

### 8.3 Perspectives of: Cellulolytic and xylanolytic activities of common indoor fungi

The selective screening of indoor fungal isolates for cellulase producers showed that three species *P. chrysogenum* and *Cladosporium sphaerospermum* would be ideal candidates. Therefore it would be interesting to do a larger scale screening of indoor *Penicillium* spp. and *Cladosporium* spp. isolates, for more potential cellulase producers. The screening should preferably be performed with several additional enzyme assays, such as filter paper activity assay and also different para-nitrophenyl substrates as these can more readily be applied in a high throughput screening setup compared to AZCL. Also an important assay to include is the application of the cellulases, which is the hydrolysis assay using a pretreated lignocellulose biomass, as it has been found that the activities observed on synthetic substrates does not correlate with the activity in hydrolysis [2].

The monoculture enzymes of the three potential isolates *P. purpurogenum*, *P. chrysogenum* and *C. sphaerospermum*, should be analyzed for hydrolysis of lignocellulose to investigate their individual potential. It should also be analyzed whether blending these crude enzymes with the crude enzymes of *Trichoderma reesei* Rut-C30 and *A. carbonarius* could result in increased hydrolysis efficiency. Additional evaluations of the selected isolates could be to co-cultivate them with *A. carbonarius* or *T. reesei* Rut-C30. Furthermore, in case the isolates yield a boosting effect in hydrolysis of lignocellulose, it could be interesting to analyze the secretome of these fungi both in monocultures and co-cultures. In this way it may be possible to identify novel enzyme activities related to lignocellulase hydrolysis. Especially if the secretome analysis show high expression of genes that have only been assigned putative activities, and if knocking out the genes result in low hydrolysis efficiency.

### 8.4 Perspectives of: Production of cellulolytic enzymes from ascomycetes; comparison of solid state and submerged fermentation

From this chapter it became clear how influential the substrate and cultivation process is for developing optimal crude enzyme blends for lignocellulose hydrolysis. Knowledge about the different ascomycetes and their respective cellulase activities show potential strategies for making crude enzyme blends that can be applied as boosters of *A. carbonarius*. Based on the activities reported for solid state fermentations (SSF), the following fungal isolates are of interest: *Fusarium clamydosporum*, *P. chrysogenum, Thermoascus aurantiacus* and *Trichoderma harzianum*. These three isolates had an overall high activitiy from cultivations

in SSF and could therefore be potential co-cultivation strains or used for mono-culture blending, if the same cultivation parameters are applied. From the activities reported in submerged fermentation (SmF) setups there were also several species that showed promise as potential boosters of *A. carbonarius* some of these were: *Aspergillus fumigatus*, *Penicillium brasilianum* and *Talaromyces cellulolyticus*. These isolates could also be analyzed by co-cultivation with *A. carbonarius* under identical SmF conditions or they could be applied as monoculture blends. Furthermore, it would be interesting to analyze whether similarly high activities can be achieved by cultivating the described fungi in different media that are more optimal for *A. carbonarius*.

Also the discussion regarding SSF being a less expensive production mechanism should be analyzed, by comparing cultivation in different SSF setups such as drum-bioreactors, tray or packed bed reactors with the classic SmF stirred tanks or airlift tanks. The comparison should be done with an emphasis on the economics of the whole process, from substrate availability (transport cost) processibility, running costs, sterilization and formulation of the product while relating it to productivity (gram cellulase/g substrate/hour or activity units /g substrate/ hour).

Based on the screening of the different cellulase activities achieved from cultivation by either SSF or SmF it would be interesting to analyze *A. carbonarius* in controlled setups of both SSF and SmF in order to determine what the optimal cultivation process for this fungal strain is. In chapter 5 preliminary results of substrate screenings and SSF growth parameters were evaluated, as will be discussed in the following section.

## 8.5 Perspectives of: Optimization of *Aspergillus carbonarius* ITEM 5010 enzyme production in solid state fermentation

The experiments performed for analysis of *A. carbonarius* enzyme production in SSF should be supplemented by analysis of all enzyme activities (β-glucosidase, xylanase, avicelase and wheat straw hydrolysis) for crude enzymes from all of the 6 media after 5, 7 and 10 days cultivations. Also for the GPW/N medium that yields crude enzymes with optimal wheat straw hydrolysis it should be analyzed whether activities could be improved by cultivation with additional water supplied during the incubation or cultivation with water saturated air. This is especially relevant for the cultivation at 30 °C where there is a higher risk of decreasing water activity by prolonged incubation.

The finding that urea and $(NH_4)_2SO_4$ were required for efficient cellulase production and possibly improved growth in GPW medium should be investigated further to also determine whether the effect was as apparent for *A. carbonarius*. In this regard it would also be interesting to perform a dedicated analysis on

the effect of different nitrogen sources when added to GPW medium. The sources that could be analyzed include corn steep liquor, sodium nitrate, yeast extract or peptone among others.

Determining the optimal cultivation process for *A. carbonarius* requires that growth in SmF be analyzed for the enzyme yield and activities. For the SmF it would here be interesting to apply the same insoluble lignocellulose waste substrates as were used in SSF, although with additional water to make a slurry. By using insoluble substrates in SmF the process becomes more comparable to the SSF although the substrate accesibility and concentration will be diluted by the additional water added to make a suspension. This approach may not be more beneficial than classic SmF where soluble substrates are applied, but it can be hypothesized that the fungi would have more surface area to attach to in the stirred suspension.

For the analysis of enzyme production in different media, determination of growth or fungal biomass would yield critical information regarding the state of the fungi. It would also provide applicable information for determining if the enzyme production follows the biomass production, and if it is better to extract enzymes during exponential growth phase or during stationary phase. Two indirect biomass determination methods were analyzed in chapter 6 and are discussed in the following section.

## 8.6 Perspectives of: Fungal enzyme production and biomass determination in solid state fermentation

For a more detailed study on ascomycetes growth in SSF systems, it would be interesting to analyze more time points, to better observe the growth rate and for information about the lag, exponential and stationary phase. This would require measurements each day during at least 10 days cultivation, including a measurement of the initial content of ergosterol (Erg) in the inoculum. Furthermore in the cultivation setup applied in this study, there was no additional water added during cultivation, which can be hypothesized to have lowered the fungal growth. This should be investigated by measuring Erg each day in both an SSF cultivation setup where water is supplied and one where it is not. It would also be possible to correlate both of these studies to enzyme production. However, for the correlation to enzyme production more para-nitrophenyl substrates should be used in order to have quantifiable enzyme activities, in contrast to the AZCL activities which are only semi-quantitative.

The Erg method, however, requires some optimization with regards to the recovery and this may be done in several ways. Firstly the liquid-liquid extraction step could be performed twice, as it was shown that a second round of pentane resulted in 2 - 20 % of extra ergosterol extracted. Secondly it is unknown whether Erg is degraded in the saponification step due to the relatively long boiling time in methanol. Alternative boiling times should be tested for improving recovery. Also it could be analyzed whether a different

extraction solvent than pentane can be used, as it was found that solubility of Erg was higher in dichloromethane. Also the sample preparation that was used entailed stopping the cultivation in order to acquire all biomass and substrate, however, it should be analyzed if the cultivation and method could be optimized so that homogenous samples could be extracted without stopping the cultivation. This has recently been shown by Steudler et al. [3] where they cultivated *Trametes hirsuta* in an SSF drum reactor, and extracted samples on 5 different days during 14 days cultivation.

Ergosterol and enzyme measurements could be used to analyze the potential of different medium compositions, by evaluating the growth of specific fungi in each setup. For lignocellulose degradation this evaluation of different media and growth regimes would provide information of the optimal enzyme production strategy which could lead to increased enzyme concentrations and therefore a lower cost.

## 8.7 Perspectives of: LPMO increase glucose and xylose yield in wheat straw hydrolysis when supplemented to crude enzymes of *Aspergillus carbonarius*

The results achieved in chapter 7 clearly showed that *Thermoascus aurantiacus* LPMO (*Ta*_LPMO) elicits much higher boosting of *A. carbonarius* wheat straw hydrolysis compared to *Podospora anserina* LPMO. As a future study it would be of great interest to create a mutant that expresses the *Ta*_LPMO. Thereby it can be determined if similar boosting can be achieved in the engineered strain or if the produced amounts are too low to elicit a boosting effect. Also with the CRISP/Cas9 system available it has become much easier for metabolic engineering, bringing with it plenty of opportunities for strain development. Besides heterologous expression of the *Ta*_LPMO it would be beneficial to attempt expression of cellobiohydrolases CBHI and CBHII from *T. reesei* Rut-C30. Other targets for metabolic engineering would be to delete genes coding for extracellular proteases or to insert genes coding for endo-1,5-α-L-arabinanase or other xylanolytic activities. Furthermore, additional auxiliary activities could be attempted expressed, such as expansin, glucooligosachharide oxidase (AA7), galactose oxidase (AA5_2) or even lignin peroxidases (AA2) or laccase (AA1_1) [4].

Additionally the strategies suggested by Kubicek et al. [5], for optimization of *T. reesei* cellulase production, could be attempted in *A. carbonarius*. For example the expression of cellobiohydrolase CBHI gene in T. reesei is significantly enhanced during cultivation in constant light, and it is regulated by the light signaling gene *env1* [5]. Therefore if a homologue of *env1* could be located in *A. carbonarius*, strains with this gene knocked out or disrupted might have improved cellulase production or at least be able to produce higher amounts of enzyme in darkness. Also the carbon catabolite repression could be attempted alleviated via disruption of the CreA gene in *A. carbonarius*, which is the orthologue of Cre1 of *T. reesei*. As a final

experiment it would be interesting to analyze whether these engineering targets could be combined with strategies for improving organic acid production, while still maintaining a stable organism. Also the improvement of the cellulase production may lead to improved growth on lignocellulosic waste, therefore in combination with improved organic acid production it would be possible to utilize the waste for sustainable production of e.g. citric, malic, fumaric or succinic acid.

The mutants growth potential in a lignocellulosic waste medium such as GPW/N (applied in chapter 5) could be analyzed by the methods evaluated in chapter 6. Furthermore it would be interesting to analyze whether *A. carbonarius* growth and production of organic acids in a GPW medium could be enhanced by addition of either purified *Ta*_LPMO or by addition of *T. aurantiacus* monoculture to the cultivation medium either at the start of the cultivation or at a later stage. By applying crude enzymes of *A. carbonarius* CBH and *Ta*_LPMO producing mutants it may be possible to achieve above 80 % hydrolysis efficiency. The resulting hydrolysate could then be used as growth medium for the organic acid producing mutants. For the hydrolysate to be applicable as growth medium, it would be necessary to analyze whether inhibitors were generated during the enzymatic hydrolysis. Furthermore there may be a possibility of using the spent hydrolysate for enzyme production by the potential CBH + *Ta*_LPMO mutant of *A. carbonarius*.

With the increased hydrolysis observed by addition of *Ta*_LPMO it should also be analyzed whether similar boosting effects could be achieved by addition of either hemicellulose degrading enzymes or by the addition of different purified CBH or endoglucanases. Also the combination of crude enzymes from *T. aurantiacus* with those of *A. carbonarius* should be analyzed both by blending monocultures and by co-cultivation of the two strains.

## 8.8 Final perspectives

Ultimately the results achieved throughout this thesis can be used for designing an application of the garden and park waste collected by Solum A/S, as the GPW/N proved a good cultivation medium for *A. carbonarius*. Therefore, it can be envisioned that an onsite enzyme production using GPW/N medium could be made, with either application of the enzymes for hydrolysis of pretreated GPW or wheat straw for organic acid production. Especially in combination with the knowledge gained for improving the hydrolysis of lignocellulose either by supplementing with novel fungal crude enzymes or by supplementing with *Ta*_LPMO. This approach might therefore lead to a sustainable production of both enzymes and possibly organic acids. It can also be speculated that several of the fungal strains that were screened in this Ph.D. study could be applied in co-cultivation on the GPW/N medium, for enhancement of the enzyme product. Directed evolution of Ta_LPMO could be attempted to improve the reactivity of this enzyme, in order to

achieve a further increase in the boosting of *A. carbonarius* crude enzymes. Finally by developing a mild pretreatment process for the GPW, it would be possible to apply this as a medium in consolidated bioprocessing using *A. carbonarius* as the main organism.

## 8.9 References


[1] Kolasa M, Ahring BK, Lübeck PS, Lübeck M. Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. Bioresour Technol 2014;169:143–8.

[2] Kabel MA, van der Maarel MJEC, Klip G, Voragen AGJ, Schols HA. Standard assays do not predict the efficiency of commercial cellulase preparations towards plant materials. Biotechnol Bioeng 2006;93:56–63.

[3] Steudler S, Bley T. Biomass estimation during macro-scale solid-state fermentation of basidiomycetes using established and novel approaches. Bioprocess Biosyst Eng 2015;38:1313–23.

[4] Levasseur A, Drula E, Lombard V, Coutinho PM, Henrissat B. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. Biotechnol Biofuels 2013;6:41.

[5] Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B. Metabolic engineering strategies for the improvement of cellulase production by Hypocrea jecorina. Biotechnol Biofuels 2009;2:19.

# Appendix I

Calibration curve for ergosterol calculation, based on blank matrix samples spiked with 7 concentrations: 1, 0.75, 0.50, 0.25, 0.10, 0.05 and 0 mg Erg/ml.

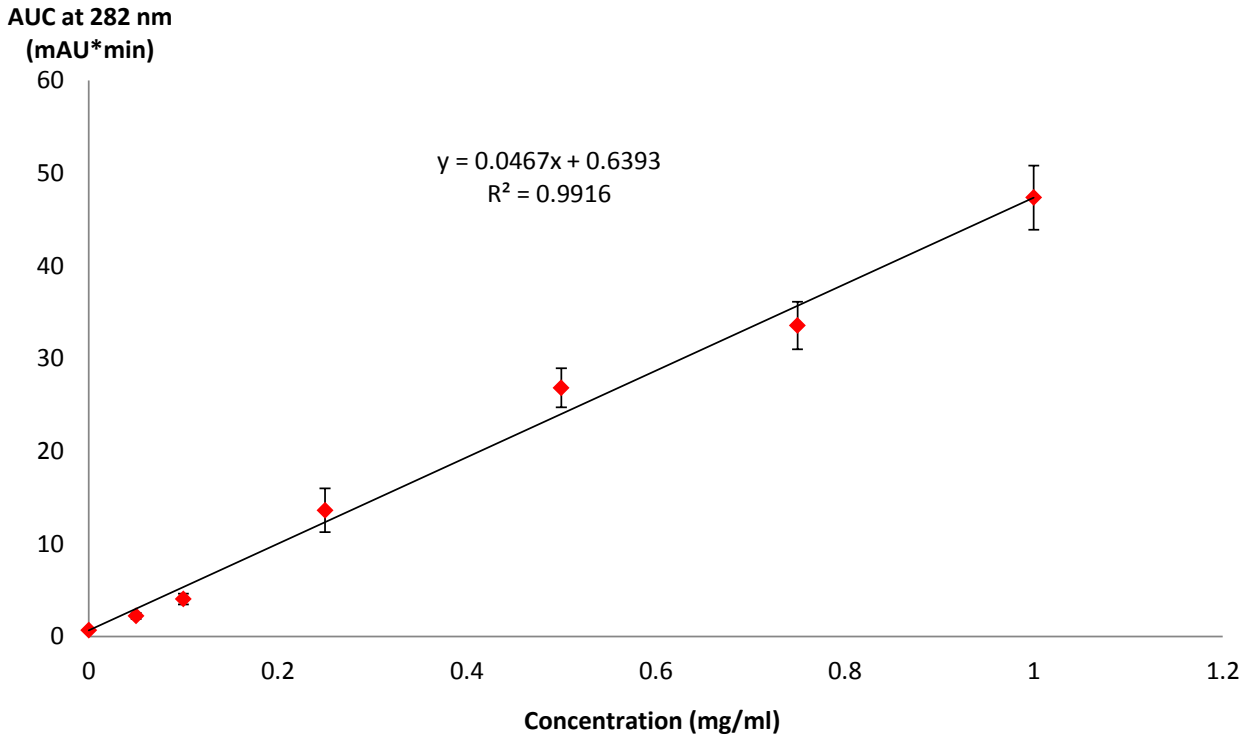


**Fig. A1** Standard curve of ergosterol area under the curve (AUC) plotted against spiked concentration in a blank WB/SP matrix. Error bars denote SD (n = 9).

# Appendix II

Recovery of ergosterol was evaluated at 0.05, 0.25 and 0.75 mg/ml from samples prepared by spiking the blank matrix before and after extraction.

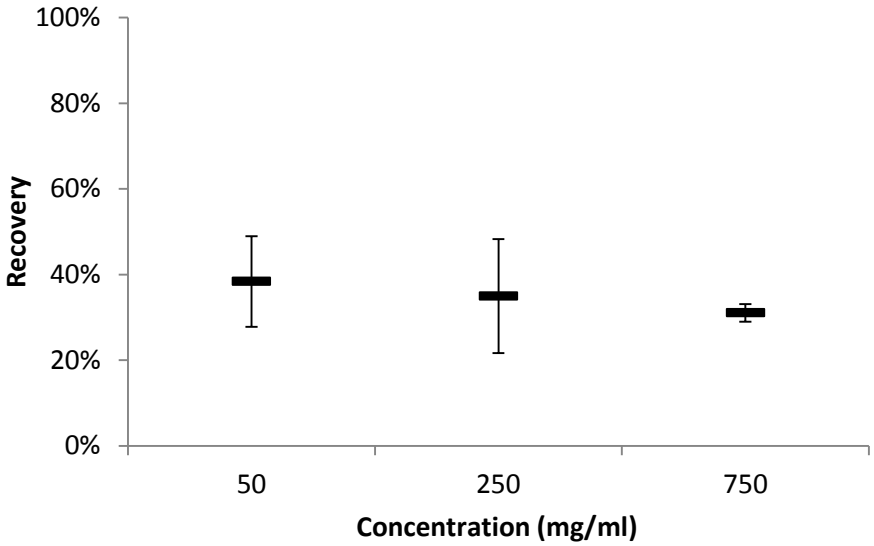


**Fig. A2** Percentage of ergosterol (Erg) recovered at three different concentrations of spiked samples before and after extraction. Error bars denote SD (n = 3).

# Appendix III

The primers that were used for construction of the vectors, pPICZα, pLPMO, pCDH and the pCAC as well as for check of correct transformants were as shown in Table A1.

**Table A1.** Sequences of primers used for vector construction.

| Construction of pPICZα vector | |
|---|---|
| Nc_CDH fwd | AGGCTGAAGCU ATGCGTACCACATCCGCTTT |
| Nc_CDH rev | AACACACU GCCAATACCAATCG |
| Pa_LPMO fwd | AGGCTGAAGCU ATGAAGTCATTTACCGCAACCG |
| Pa_LPMO rev | AACGCATU GATGATACCATTGATTA |
| Construction of pLPMO, pCDH vector | |
| pCoxA-L1- fwd | AGACTTCGU ACGACGGCATTTGAGCAACA |
| pCoxA rev | ATGACTTCAU TGTCCTGGTGGGTGGGTTG |
| LPMO fwd | ATGAAGTCAUTTACCGCAACCG |
| LPMO rev | ATTACACTU TTAAACGCATTGATGATACCATTGA |
| Nig-trpC-fwd | AAGTGTAAU CAAACATGGGATTTAAGGGCAT |
| Nig-TtrpC-L3-rv | ATTACACTU CCCGTATCCATTGGGGTATTTTGTCCG |
| PgpdA-L1-fwd | ACGAAGTCU GCGTAAGCTCCCTAATTGGC |
| PgpdA-rv | ATGTGGUACGCAU TGTGATGTCTGCTCAAGCGG |
| CDH-fwd | ATGCGUACCACAUCCGCTTTC |
| CDH-rv | AATAGACCU CTAAACACACTGCCAATACCAATCG |
| Nid-TtrpC-fwd | AGGTCTATU TGATTTAATAGCTCCATGTCAACAAG |
| Nid-TrpC-L4-rv | AGGTCTATU GAGCCAAGAGCGGATTCCTCAGTCTCG |
| L2-fwd | AAGTCTACU TGCCTCCATAGCCCCTCC |
| L2-rev | AGTAGACTU TTACCTCTAAACAAGTGTACCTGTGC |
| PgpdA-HPH,L3-fwd | AAGTGTAAU TAAGCTCCCTAATTGGCCC |
| L4-fwd | AGGTCTATU GAGCCAAGAGCGGATTCCTC |
| Construction of pCAC | |
| PgpdA-pac-up-fwd | GGGTTTAAU GCGTAAGCTCCCTAATTGGC |
| TtrpC-short-pac-dw-rv | GGTCTTAAU GAGCCAAGAGCGGATTCCTC |
| gRNA-PS4-rv | AGCTTACUCGTTTCGTCCTCACGGACTCATCAGAGTGGGCGGTGATGTCTGCTCAAGCG |
| gRNA-PS4-fwd | AGTAAGCUCGTCAGTGGGATCTCAAGAACTACGTTTTAGAGCTAGAAATAGCAAGTTAAA |
| Check primers | |
| AlbA fwd (negative control) | TGGTGGTGTGGAGGCTACGA |
| AlbA rev (negative control) | TGACATCGCACACATCCAATCC |
| AlbA+pLPMOpCDH fwd | TGGACCGATGGCTGTGTAGA |
| AlbA+pLPMOpCDH rev | ATCCAAGGTTTGACTGGGGAA |

# Appendix IV

The SDS-PAGE analysis of purified *P. anserina* LPMO and *N. crassa* CDH is shown in Fig. A3.

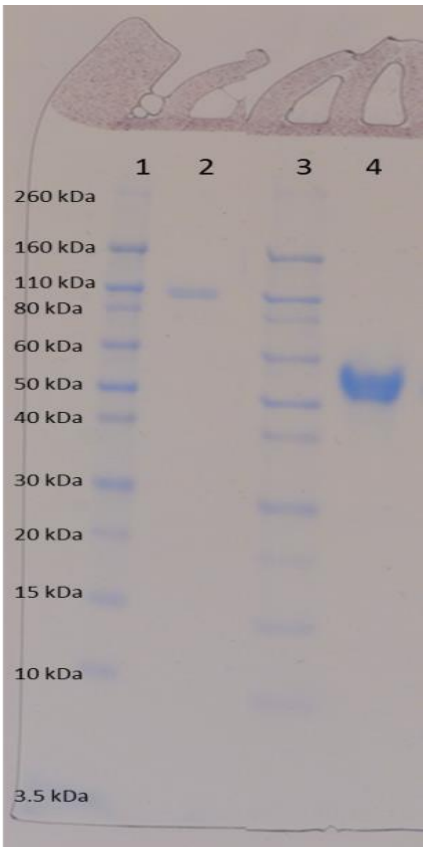


**Fig. A3** SDS-Page of purified CDH (lane 2) and LPMO (lane 4), with Novex Sharp Prestained protein ladder (lanes 1 and 3).

# Appendix V

Presented below are the three hits for LPMO's (AA9) in *Aspergillus carbonarius* ITEM 5010 v3 genome:

**Hit no. 1**

64814|estExt_Genewise1Plus.C_41408:

MSLSKVASVLLASFSLVAGHGYVSSIEVDGTTYGGYLVDTYYYESDPPELIAWSTNATDDGYVSPTNYDSSNIICHRGSSPGA
LSAPVAPGGTVKMTWNTWPDDHHGPVITYLANCNGPCSDVDKTALQFFKIDAGGLIDDTDIPGTWASDQLIDDSYSRTITI
PTDIEAGNYVLRHEIIALHGAEDLDGAQNYPQCINLNVTGSGTATPSGTLGTALYKDTDPGIYVDIWQTISSYTIPGPTLYTAG
RTATATAAAVTTTAALTSASTTEAAVMTSAAVSSASSHTTMITSSRPQPTGSANTDTTDAQTGSGPTTVGSQPQPDMTGS
STTTTSSASPATTSDVLSGACSQEDYWYCNNGTAFQRCVNGEWDASQNMAAGTECTAGISQTLTISASSKRRDIGRVRRR
HV

**According to CDD: This is a GH61 (AA9)**

**InterPro: Confirms it as a GH61 (AA9)**

**Hit no. 2**

jgi|Aspca3|210317|fgenesh_isotigs_kg.15_#_151_#_isotig07135

MNQIKSIIHLTLLSAATRVAAHGHVSNIVINGVSYRGWDINSDPYNSDPPVVVAWQTPNTANGFISPDAYDTDDIICHLDAT
NARGHAVVAAGDKISLQWTTWPDSHHGPVISYLANCGSSCESVDKTTLEFFKIDGVGLVDESEVPGIWGDDQLIADNNWL
VEIPNVAPGYYVLRHELIALHGAASENGAQNYPQCFNLQITGSGTAEPSGVLGTELYTPTDAGILVNIYQSLSYEIPGPTLIPEA
VSVVQSSSTITASGTPVTGTATSTAT

**According to CDD: This is a GH61 (AA9)**

**InterPro: Confirms it as a GH61 (AA9)**

**Hit no. 3:**

jgi|Aspca3|57612|e_gw1.19.216.1 – no hits in proteomics data

MRHAQSASLLTALLSATQVAAHGHVTNIVVDGVYYEGFDINSFPYESDPPKVAAWTTPNTGNGFISPDEYRNPNIICHENA
TNAQAHVVVGAGEKVNIQWTAWPDSHHGPVLDYLANCGDNCETVDKTTLEFFKIDGVGLVSDTEVPGTWGTDQLINNN
NSWLVEIPPSIAPGNYVLRHELIALHSAEEEDGAQNYPQCFNLQVTGTGTATPSGVLGTELYTATENGILVNIYSTMSTYTVP
GPSQYSGAVSVTQTTSAITSTGTAVTGSATASASAVATASSSVAAATSAADSTSAAEPTITSAPAVTSAAAGSSGTQSLYGQC
GGINWSGATSCASGSSCHSYNPYYYQCIASA

**According to CDD: This is a GH61 with a CBM**

**InterPro: Confirms it as a GH61 with CBM**